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Clinical and Experimental

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STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

I. Experimental Peritonitis

George R. Kingsley, M.S.,* and L. A. Terzian, Ph.D.†
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A PREVIOUS report on patients with liver disease[1] established definite differences in the results of determinations of serum proteins by biuret and Kjeldahl methods. A further comparison of these methods was made in experimental peritonitis in dogs to confirm previous findings in similar diseases in human individuals. As indicated previously, it is not known whether the changes observed represent a gain or a loss of nitrogen or of peptide linkages. For convenience they are described in terms of net change of biuret reactivity.

METHODS

All protein determinations in this investigation were made simultaneously by the macro-Kjeldahl and biuret methods,[2, 3] using the ether-sodium sulfate method[4] for the separation of albumin and globulin. Fresh, unhemolyzed serum from blood samples taken either by vein or cardiac puncture was used. Adult mongrel dogs weighing an average of 16 kilograms were used. Prior to operation, small doses of morphine and atropine were administered subcutaneously. Ether anesthesia and aseptic technique were employed. Spreading peritonitis was induced according to the procedure described by Bower, Burns, and Mengle.[5] The appendix and its mesentery, together with all its blood vessels, were ligated and the appendix was traumatized. Sixty to 90 ml. of castor oil, varied according to the weight of the animal, were administered by a catheter immediately following operation. Water was withheld for forty-eight hours to avoid persistent vomiting. Simple laparotomys were performed on control animals exposing the viscera without ligation of blood vessels or traumatization of the appendix following the same anesthetization as in the experimental animals. Plasma volume and blood volume were estimated according to the method outlined by Gibson and Evans.[6]

EXPERIMENTAL

Induced spreading peritonitis in dogs apparently caused an average decrease of biuret reactivity of globulin of 0.66 Gm. per cent (Table I), with a standard error of ± 0.08. This value is highly significant. Nine out of ten animals showed a significant difference in serum globulin as determined by biuret and Kjeldahl methods. Apparently the change occurs soon after the development of peritonitis, since this difference was fairly uniform from the

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TABLE I. DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS BEFORE AND 2 TO 10 DAYS AFTER SIMPLE LAPAROTOMY IN 3 DOGS AND SPREADING PERITONITIS IN 10 DOGS

	DIFFERENCE, KJELDAHL MINUS BIURET			
	BEFORE OPERATION		AFTER OPERATION	
	ALB. (GM. %)	GLOB. (GM. %)	ALB. (GM. %)	GLOB. (GM. %)
Standard deviation, laparotomy	−0.13	0.17	0.13	−0.13
Standard deviation, peritonitis	0.07	0.06	0.04	0.66
2.6 × std. dev., laparotomy	±0.34	±0.44	±0.34	±0.34
2.6 × std. dev., peritonitis	±0.18	±0.16	±0.10	±1.72

Number of dogs with changes in serum albumin and globulin after operation greater than 2.6 times the standard deviation (elevated, +; no significant change, 0; reduced, −)

	ALBUMIN			GLOBULIN		
	+	0	−	+	0	−
Laparotomy	0	3	0	0	3	0
Peritonitis	1	8	1	9	1	0

second to the tenth day following the operation. No significant change occurred in the albumin biuret reactivity or total N during the same period. Average differences between protein determined by biuret and Kjeldahl N methods in both albumin and globulin were within normal limits before peritonitis was induced, and similarly, average differences in the control (laparotomy) animals were not significant. In spite of a large loss of 8 to 31 per cent (18.9 per cent average) of total circulating plasma protein, an increase of 0.4 Gm. per 100 ml. was observed in total protein by the Kjeldahl determination, probably as a result of dehydration. However, the biuret determination indicated no change in total protein before and after peritonitis. The Kjeldahl determination indicated a decrease in A/G ratio from 1.66 to 1.09, while a smaller decrease from 1.67 to 1.39 in A/G ratio was shown by the biuret method.

DISCUSSION

It has been shown[1] that liver disease will cause changes in the normal relationships between total N and biuret N in serum proteins, especially globulin. The degree of loss of biuret N in globulin in experimentally induced peritonitis is similar to that observed in cirrhosis. A comparable change in experimental peritonitis after two to ten days may be due in part to the rapid loss of large amounts of circulating plasma protein which placed an additional burden on a liver already actively involved and further impaired by the toxemias of the disease. Since these dogs ate little during the acute phase of the infection, malnutrition may have been an additional factor.

SUMMARY

1. Induced spreading peritonitis in dogs produced differences in serum globulin determinations by the biuret as compared with total nitrogen methods. These differences were similar to those observed in cirrhosis.

2. No corresponding changes in the albumin fraction were observed.

REFERENCES

1. Kingsley, G. R.: Differences Between Biuret and Kjeldahl Methods for Serum Protein Determinations in Chronic Liver Disease, J. Biol. Chem. **140**: lxix, 1941.
2. Kingsley, G. R.: Direct Biuret Method for Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, J. LAB. & CLIN. MED. **27**: 840, 1942.
3. Kingsley, G. R.: The Determination of Serum Total Protein, Albumin and Globulin by the Biuret Reaction, J. Biol. Chem. **131**: 197, 1939.
4. Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin, J. Biol. Chem. **133**: 731, 1940.
5. Bower, J. O., Burns, J. C., and Mengle, H. A.: Spreading Peritonitis Complicating Acute Perforative Appendicitis; Experimental Studies, Arch. Surg. **37**: 751, 1938.
6. Gibson, J. G., Jr., and Evans, W. A., Jr.: Clinical Studies of Blood Volume; Clinical Application of Method Employing Azo Dye "Evans Blue" and Spectrophotometer, J. Clin. Investigation **16**: 301, 1937.

STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

II. Effect of Occlusion of the Hepatic Artery and Ligation of the Gastroduodenal Artery on Serum Proteins

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A PREVIOUS report on patients with various diseases[1] and one on animals[2] established definite differences in the results of determinations of serum proteins by biuret and Kjeldahl methods. Further comparison of these methods was made following occlusion of the hepatic artery and ligation of the gastroduodenal artery in dogs to determine the effect of a disturbance of arterial circulation and nutrition of the liver upon the biuret and Kjeldahl N relationship in serum proteins and in addition to establish the relationship of this liver impairment to previous findings in liver disease.

METHODS

All protein determinations were made simultaneously by the macro-Kjeldahl and biuret methods.[3, 4] The ether-sodium sulfate method[5] was used for the separation of albumin and globulin. Only fresh unhemolyzed serum was used for all determinations.

Sodium Amytal intraperitoneally was used for anesthesia. A partial clamp was applied to the hepatic artery which was gradually tightened from one to eight minutes during the two or three weeks following the operation on three dogs. Cellophane was placed and held by two to three Michel skin clips around the hepatic artery to produce occlusion in fourteen other dogs. The gastroduodenal branch of the hepatic artery of all dogs was double ligated and divided at the time of operation. Ten or more weeks later the hepatic artery was ligated at the point of occlusion. Repeated determinations of serum proteins (biuret and total N), urea, nonprotein nitrogen uric acid, hemoglobin, sugar, cholesterol, and cholesterol ester were made on the blood of the dogs for periods up to 120 days following the initial operation.

RESULTS

In every dog in the periods studied (Table I) a loss of biuret reactivity occurred in the globulin fraction. The average loss of biuret N in the globulin fraction from the second to the one hundred and thirtieth day after the operation was 0.36 Gm. per cent (Fig. 1, Table I).

There was a gradual return to normal in the biuret N/Kjeldahl N ratio of the globulin following the operation from a loss of 0.40 Gm. per cent biuret N to 0.08 Gm. per cent on the thirty-fifth day. Losses occurred again until 0.58 Gm. per cent globulin biuret N had been reached on the fifty-fifth day, after which a slow recovery occurred until the one hundred thirtieth day when a smaller loss of biuret N of 0.30 Gm. per cent was reached. No average significant gain or loss in albumin biuret N occurred in the 130 days following the

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operation (Table I). However, the albumin values represented digrammatically in Fig. 1 show an irregular loss of albumin biuret N in the first thirty-five days following the operation from 0.1 Gm. per cent to –0.20 per cent.

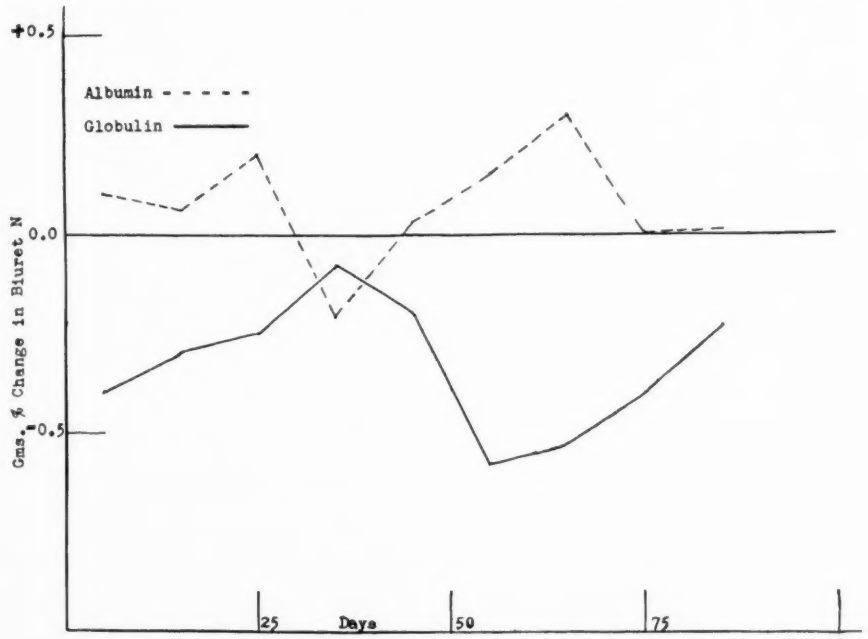

Fig. 1.—Diagrammatic representation of gain and loss of biuret linkages (Gm. % N) of serum proteins in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table I).

TABLE I. DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS FOLLOWING OCCLUSION OF THE HEPATIC ARTERY AND LIGATION OF THE GASTRODUODENAL ARTERY

NUMBER OF DOGS	DAYS AFTER OPERATION	BIURET		DIFFERENCES, BIURET MINUS KJELDAHL	
		ALB. (GM. %)	GLOB. (GM. %)	ALB. (GM. %)	GLOB. (GM. %)
10	2	3.7	2.1	0.1	–0.4
7	3-10	3.3	2.2	0.1	–0.4
11	11-30	3.0	3.4	0.1	–0.3
10	31-50	2.6	3.2	–0.1	–0.1
8	51-70	3.0	2.9	0.2	–0.6
13	71-90	2.9	3.0	0.0	–0.4
8	91-130	3.2	3.1	0.0	–0.3

Albumin biuret N increased from the thirty-fifth day to the sixty-fifth day when a gain of 0.30 Gm. per cent was shown. The biuret N/Kjeldahl N ratio returned to normal on the seventy-fifth to eighty-fifth days. The greatest average loss of albumin biuret N occurred on the thirty-fifth day. There was an apparent relationship between the simultaneous gain of albumin biuret N and loss of globulin biuret N, and loss of albumin biuret N and gain of globulin biuret N as shown in Fig. 1. The highest globulin biuret N occurred at the

time of the greatest loss of albumin biuret N, and the reverse occurred when the greatest loss of globulin biuret N was reached at the approximate time of the greatest increase in albumin biuret N. A few animals, Dogs 405 and 427 (Fig. 2) and Dogs 479 and 723 (Fig. 3), showed marked reversal in their loss and gain of biuret N in albumin and globulin. A gain of globulin biuret N

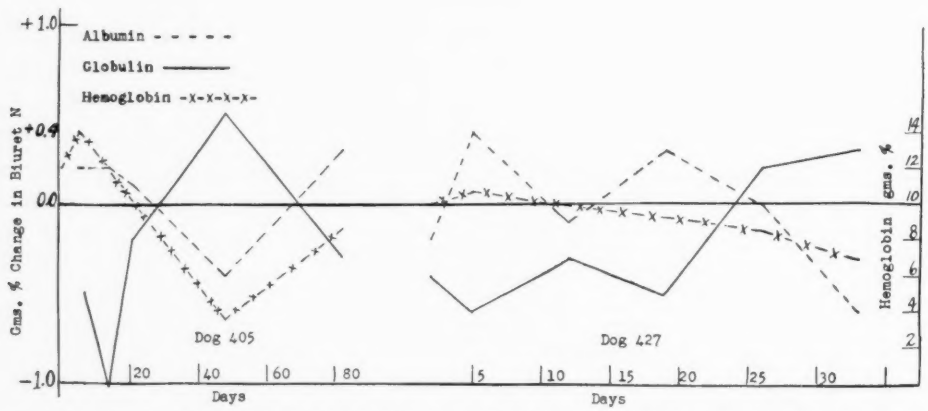

Fig. 2.—Diagrammatic representation of gain and loss of biuret linkages (Gm. % N) of serum proteins and hemoglobin in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table I).

was reflected in a simultaneous loss of albumin biuret N, and loss of globulin biuret N occurred when there was a gain of albumin biuret N. This direct relationship was continuous throughout all periods studied after the dogs were operated upon.

Hemoglobin levels had a direct quantitative relationship to the biuret changes in albumin and globulin, as shown in Dogs 405 and 427 (Fig. 2). Loss of albumin biuret N accompanied loss of hemoglobin, and an increase in hemoglobin occurred with a gain in albumin biuret N. However, only these two dogs had abnormally low hemoglobin levels.

All dogs showed no significant change in whole blood urea N, nonprotein nitrogen, uric acid, glucose, and serum cholesterol and ester. Only a few dogs showed slightly lowered hemoglobin levels.

All dogs were examined at autopsy, and sections of the liver, spleen, and arteries were taken. Histologic findings are to be reported elsewhere. The hepatic artery was completely occluded except in three dogs in which occlusion was almost complete.

DISCUSSION

The most persistent change in the serum proteins of dogs following occlusion of the hepatic artery and ligation of the gastroduodenal artery is the loss of globulin biuret N. This is also the most typical change in the serum proteins of patients with liver disease.[1] This similarity would indicate that impairment in arterial circulation and nutrition of the liver produces structural changes within a relatively short time in the serum proteins which are observed in cirrhosis after much more time has elapsed. The large loss of globulin biuret

N immediately after the operation in the dogs (Fig. 1) probably results from the ligation of the gastroduodenal artery. Apparently collateral circulation to replace that of the gastroduodenal artery is established in the first thirty-five days following the operation since the globulin biuret N returned to almost normal. However, there is a gradual loss of albumin biuret N during this time. This inverse relationship of the biuret N of albumin and globulin is

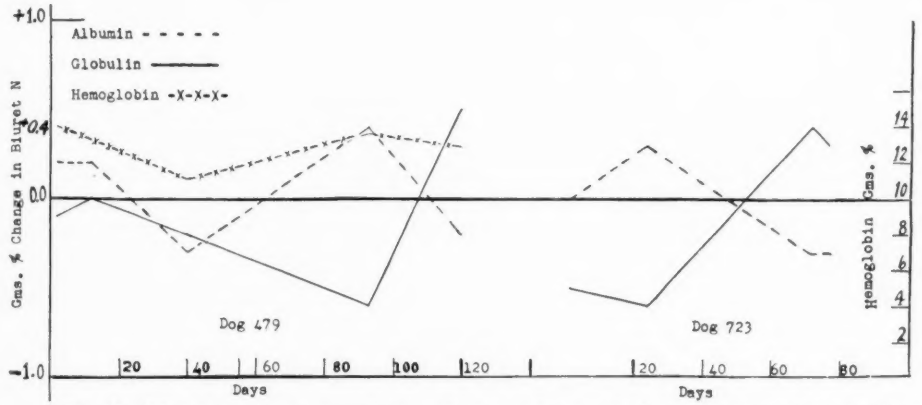

Fig. 3.—Diagrammatic representation of gain and loss of biuret linkages (Gm. % N) of serum proteins and changes of hemoglobin in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table I).

very clearly shown in individual Dogs 405 and 427 (Fig. 2) and 479 and 723 (Fig. 3). These changes observed in the thirty-five days following the operation would primarily result from the malnutrition produced by the loss of gastroduodenal arterial circulation. The increasing loss of globulin biuret N accompanied by increase of albumin biuret N after the thirty-eighth day following the operation (Fig. 1) may have resulted from the gradual occlusion of the hepatic artery. This change increased until about the sixty-fifth day when greater loss of globulin biuret N and gain of albumin biuret N were reached. The rate of occlusion of the hepatic artery was variable in the dogs. Some dogs examined showed complete occlusion of the hepatic artery after thirty-five days. After the sixty-fifth day the albumin and globulin biuret N again started to return to normal, probably as a result of establishment of collateral hepatic arterial circulation. Nearly every period studied in each dog showed a total net loss of biuret N. This is also true in induced peritonitis in dogs[2] in which the principle loss of biuret N occurs in the globulin. The tendency for the globulin fraction to be more labile to greater biuret N changes than albumin may be due to its larger molecular size which requires a more highly developed complex biologic process for its formation and maintenance. The biuret linkages in globulin may be less stable. The formation of globulin also may be secondary to that of albumin. However it is interesting that the approach to normal biuret N in albumin and globulin occurs at the same time (Figs. 1, 2, and 3) and that their graph lines of a gain or loss of biuret N nearly always cross at 0.1 per cent biuret N. This would indicate that there is some definite relationship between

albumin and globulin in the mode of formation, origin, or exchange of their biuret N. There is also some indication that hemoglobin concentration has some relationship to gain and loss of biuret N in albumin and globulin (Dogs 405 and 427, Fig. 2). The greatest loss of albumin biuret N and the greatest gain of globulin biuret N is accompanied by the lowest hemoglobin levels. Since loss of albumin biuret N usually occurred only when the A/G ratio was 1 or less, it would appear that quantitatively albumin and globulin levels have some relationship to the maintenance of normal biuret N/Kjeldahl N ratio of serum proteins.

SUMMARY

1. Disturbance of the metabolism of the liver either by occlusion of the hepatic artery or ligation of the gastroduodenal artery in dogs is followed by a large loss of serum globulin biuret N and a smaller gain of serum albumin biuret N, resulting in a net loss of biuret N in the serum proteins.

2. No significant change in whole blood urea N, nonprotein nitrogen, uric acid, glucose, and serum cholesterol and ester was observed in dogs following occlusion of the hepatic artery and ligation of the gastroduodenal artery. Low hemoglobins were observed in a few dogs.

REFERENCES

1. Kingsley, G. R.: Differences Between Biuret and Kjeldahl Methods for Serum Protein Determinations in Chronic Liver Disease, J. Biol. Chem. **140**: lxix, 1941.
2. Kingsley, G. R., and Terzian, L. A.: Studies of the Differences Between Biuret and Kjeldahl Determinations of Serum Proteins. I. Experimental Peritonitis, J. LAB. & CLIN. MED. **34**: 1175, 1949.
3. Kingsley, G. R.: Direct Biuret Method for Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, J. LAB. & CLIN. MED. **27**: 840, 1942.
4. Kingsley, G. R.: The Determination of Serum Total Protein, Albumin and Globulin by the Biuret Reaction, J. Biol. Chem. **131**: 197, 1939.
5. Kingsley, G. R.: A Rapid Method for the Separation of Serum, Albumin and Globulin, J. Biol. Chem. **133**: 731, 1940.

STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

III. Liver and Other Diseases

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PRELIMINARY reports on patients with chronic liver disease,[1] peritonitis in dogs,[2] and occlusion of the hepatic artery and ligation of the gastroduodenal artery in dogs[3] established definite differences in the results of determinations of serum proteins by biuret and total nitrogen (Kjeldahl) methods. Since good agreement between these methods exists in normal individuals,[4] further comparison was made in order to ascertain the significance and possible diagnostic value of such differences in various diseases. The changes observed indicate that either gains or losses of nitrogen or of biuret linkages have occurred. The data at present do not permit a distinction to be made.

METHODS

All protein determinations in this investigation were made simultaneously by the macro-Kjeldahl (using factor 6.25 to convert N to protein) and biuret methods.[4, 5] The ether-sodium sulfate method[6] was used for the separation of albumin and globulin. Only fresh and unhemolyzed serum was employed. Urea and nonprotein nitrogen determinations were made to correct the Kjeldahl protein determinations for nonprotein nitrogen. The Kjeldahl digestion for total protein determination was carried out by gently boiling in a 300 ml. Kjeldahl flask 10 ml. of concentrated sulfuric acid, 4 Gm. of sodium sulfate and 0.5 Gm. of copper sulfate with 1 ml. of serum for one hour. Twelve milliliters of albumin-sodium sulfate solution from the globulin precipitation, equivalent to 0.8 ml. of serum, were used for the albumin determination. The same amounts of reagents were used as in the total protein determination with the exception of the addition of only 1.5 Gm. of sodium sulfate. No increase in total nitrogen was observed when digestion was continued for two, four, and six hours.

RESULTS

The data obtained from the study of various types of liver disease have been classified (Table I) as Laënnec's cirrhosis, Banti's syndrome, biliary or obstructive, and miscellaneous hepatomegalies. The data of this table confirmed previous observations[7-11] with regard to decreased albumin and correspondingly decreased A/G ratio. Differences between the results of biuret and Kjeldahl determinations in the various types of liver disease were very similar. There was a consistent decrease of peptide linkages as measured by the biuret reaction (or increase in total nitrogen) of the globulin fraction in all types of liver diseases studied. The average loss of normal biuret reactivity of the globulin fractions of the eighty-four patients with liver disease studied was 16 per cent. The

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TABLE I. DIFFERENCE BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS IN VARIOUS HUMAN DISEASES

CLINICAL CONDITION	ALBUMIN						GLOBULINS					
	MEAN		STANDARD ERROR	NUMBER OF INDIVIDUALS WITH KJELDAHL MINUS BIURET N GREATER THAN T*			MEAN		STANDARD ERROR	NUMBER OF INDIVIDUALS WITH KJELDAHL MINUS BIURET N GREATER THAN T*		
	BIURET (GM. %)	KJELDAHL (GM. %)		+	0	−	BIURET (GM. %)	KJELDAHL (GM. %)		+	0	−
Normal[4]	5.28	5.28	±0.02	0	15	0	2.08	2.13	±0.04	0	15	0
Diseases of liver												
Laënnec's cirrhosis	3.80	3.72		2	11	6	2.78	3.26		15	4	0
Banti's syndrome	3.90	3.68		0	4	4	2.30	2.92		7	1	0
Gall bladder disease	4.00	3.98		5	18	8	2.55	3.04		24	6	1
Liver enlargement	4.31	4.30		3	12	15	2.33	2.72		12	7	1
Carcinoma	3.81	3.80		0	5	1	2.58	2.79		2	3	1
All liver diseases	4.00	3.96	±0.12	10	50	24	2.53	3.00	±0.10	60	21	3
Thyroid disease	4.48	4.37	±0.05	1	9	3	2.11	2.54	±0.24	9	4	0
Diseases of blood	4.23	4.15	±0.26	3	8	6	2.95	2.74	±0.39	5	2	10
Typhoid fever	3.75	3.73		0	3	1	2.55	3.03		4	0	0
Typhoid recovered	4.50	4.58		0	4	0	2.55	2.68		1	3	0
Miscellaneous diseases				13	29	9				19	18	14

*$T = \frac{\text{Average difference}}{\text{Standard error}}$, where P = 0.01.

average difference of 0.47 grams per cent between protein determined by Kjeldahl and biuret methods in the globulin fraction was highly significant as the standard error of this difference was only ±0.10.

This difference between Kjeldahl and biuret determinations of the globulin fraction was reflected in a corresponding difference of Kjeldahl and biuret N in the total protein determinations which resulted in a significantly higher A/G ratio as determined by the biuret method. On the average, there was no significant increase in the biuret reactivity of the albumin, although there appeared to be a tendency for an increase in twenty-four cases. In the group of eighty-four cases of hepatic disease, only three, one of chronic cholangitis, one of scurvy with hepatomegaly, and one of metastatic neoplasm, exhibited a significant increase in biuret linkages of the globulin fraction.

Toxic thyroid disease (Table I) was very similar to portal cirrhosis in the abnormal biuret reactivity/Kjeldahl N ratio of the globulin fraction. A decrease of 17 per cent in the biuret linkages of the globulin fraction occurred. However, as the number of cases in this group was small, the standard error of the differences of the Kjeldahl and biuret determinations of globulin and A/G ratio was not as highly significant as in the much larger group of liver diseases. The increase of biuret linkages of the albumin fraction even though small was much more significant than that found in portal cirrhosis. The total protein and A/G ratio as determined by the Kjeldahl method were fairly normal, the A/G ratio being the highest of any group studied. This demonstrates that in disease, serum proteins may be normal quantitatively by Kjeldahl determinations when they are actually abnormal in their biuret linkages. The finding of a small significant change in the albumin fraction in thyrotoxicosis together with a large loss of biuret linkages in the globulin fraction substantiates the evidence[12, 13] presented for the activity of the thyroid in the formation of serum globulin. However, secondary liver damage may follow thyroid hyperactivity.

A majority of the various types of anemias, diseases of the blood (Table I), as individual cases showed an increase in biuret linkages in both protein fractions. This group on the average showed no significant increase in biuret linkages, probably because of the great range of individual values (globulin 1.4 to 5.4) and because of the different types of anemias in the small group. However, if the gain and loss of biuret linkages in serum albumin and globulin are added, a significant net gain in biuret linkages is observed, the average gain being 0.3 Gm. per cent, with a standard error of ± 0.12 per cent. This is the only group studied which showed an over-all gain in biuret linkages. These data indicate that a disturbance of the blood cells may act as a stimulus for the temporary overproduction of biuret linkages in serum albumin and globulin.

Serial determinations of biuret linkages (Table I) were made in four cases of typhoid fever from the second and fourth to the eighth and twelfth week of the disease. Little change occurred in the albumin fractions except in one case complicated by hypertension. In all, the globulin fraction showed a loss of biuret linkages starting with the fourth week and returned to normal with clinical recovery. The loss of biuret linkages in globulin resembled that in

portal cirrhosis and may indicate at least temporary liver damage in typhoid. An indication also was given that the liver's ability to form normal proteins was noticeably impaired until after the third week of illness.

A large number of miscellaneous diseases, some of which showed significant changes in the distribution of biuret linkages in the protein fractions, are summarized in Table I. A case of malnutrition with transfusion reaction and another transfusion reaction showed a marked loss of biuret linkages in the albumin fraction. Diabetes, diabetic coma, and complicated diabetes showed wide variations in distribution of biuret N. Two cases of cystinuria showed a simultaneous loss of biuret N in the globulin and gain in the albumin fractions. A case of chronic ulcerative colitis resembled malnutrition in biuret N changes as did nutritional edema. A patient with myxedema showed a marked increase in biuret N in the globulin with no change in the albumin. This is in contrast to the decrease in biuret N in the serum globulin of thyrotoxicosis. A patient with dystonia quadriplegia showed an almost equal exchange of biuret N from globulin to albumin, a loss and gain of about 20 per cent. A case of schizophrenia showed an opposite transfer. However, this patient was suspected of having hepatic disease, although no definite diagnosis could be made. One patient with lymphogranuloma inguinale, who showed marked inversion of A/G ratio, exhibited changes in biuret N similar to those in portal cirrhosis. In one patient with lymphopathia venereum there was an increase in biuret linkages of the globulin fraction, while two others showed a decrease; all three of these patients had definite hyperglobulinemia. Patients suffering from cardiac decompensation, acute nephritis, myoma uteri, and bronchopneumonia showed no changes in the normal distribution of biuret N.

CASE REPORTS

A few interesting cases exhibiting reversal of biuret and Kjeldahl N differences coinciding with marked clinical changes were studied over a period of several weeks. A colored man, 30 years old (No. 1, Table II), with an apparently quiescent sickle cell anemia showed about 20 per cent gain in biuret linkages of the globulin fraction. There was also a 16 per cent loss of biuret linkages in the albumin fraction. About two weeks later, the distribution of biuret reactivity in the protein fractions had returned to normal. However, this condition was not stabilized. A marked reversal of biuret distribution occurred four weeks later resulting in a 26 per cent loss of biuret reactivity in the globulin fraction and a small increase of biuret N in the albumin fraction. During these six weeks the icterus index had increased from 7 to 450, urea N from 14 to 23 mg. per 100 c.c., while cholesterol ester decreased to 1 per cent of the total cholesterol. Although the total protein was elevated with a low A/G ratio, of 0.7 to 0.8, there was little significant quantitative change in the protein fractions throughout the six-week period. This again shows that marked qualitative changes in the proteins may take place while no quantitative changes occur.

A 6-year-old girl, five months after second degree burns (No. 2, Table II) exhibited changes in biuret reactivity of serum proteins similar to those observed in portal cirrhosis. Six to eight weeks later, coincident with malnutrition, fever, anemia, and urinary tract infection, a reversal of biuret nitrogen distribution had occurred with a loss in albumin and a gain in globulin biuret linkages. Eleven weeks later after a diet rich in amino acids, proteins and vitamins, the patient showed marked clinical improvement and the biuret distribution had returned to normal. However, seven weeks later the patient returned to the hospital with malnutrition, fever, and anemia and again exhibited abnormal biuret distribution typical of this clinical change.

TABLE II. REVERSAL OF KJELDAHL MINUS BIURET DIFFERENCES IN DISEASED INDIVIDUALS

NO.	CLINICAL CONDITION	CHANGES IN KJELDAHL PROTEIN MINUS BIURET PROTEIN (GM. %) WITH TIME						
1	Sickle cell anemia	Albumin	0.5	0.0	0.2	0.5		
		Globulin	-0.9	-1.0	1.2	0.2		
		Date	3/10	3/26	4/23	5/1		
2	Burns	Albumin	-0.3	-0.5	0.7	-0.3	0.9	
		Globulin	0.7	0.2	-0.2	0.2	-0.3	
		Date	3/25	4/10	4/23	7/11	9/27	
3	Multiple myeloma	Albumin	0.3	0.0	0.2	0.2		
		Globulin	-0.4	-0.6	0.3	0.8		
		Date	7/11	7/14	9/25	10/9		
4	Biliary cirrhosis	Albumin	0.0	-0.1	0.1	0.1		
		Globulin	1.2	1.4	1.0	1.5		
		Date	10/7	10/21	11/7	11/25		
5	Nephrotic syndrome	Albumin	0.2	0.7	0.6	0.2	0.5	0.4	0.2
		Globulin	0.2	-0.5	-0.4	0.0	-0.2	-0.6	0.0
		Date	5/12	5/14	5/15	5/16	5/20	5/21	6/4

A patient with multiple myeloma with pathologic fracture of the femur (No. 3, Table II) and a large increase in globulin showed an increase in biuret linkages in the globulin fraction following the fracture. Three months later, a large decrease in biuret reactivity in the globulin occurred. Qualitatively and quantitatively the albumin remained fairly constant throughout the period of investigation. It is interesting to note that a quantitative globulin increase was accompanied by an increase in biuret reactivity in this case, and a fall of total globulin to normal level was accompanied by a decrease in biuret reactivity.

No. 4 (Table II) illustrates in biliary cirrhosis the constancy with which a large loss of biuret reactivity occurred in the globulin fraction over a period of several weeks with little change in albumin. Administration of bile salts for twenty days had no apparent effect on the proteins.

Simultaneous biuret and Kjeldahl determinations were carried out before several plasma transfusions in a patient presenting the symptoms of a nephrotic syndrome (No. 5, Table II). Although the control for biuret linkages of albumin and globulin serum fractions before the first transfusion was within normal limits, the biuret linkages of the albumin decreased and those of the globulin increased twenty-three to thirty hours following transfusion. The net change as shown in total protein biuret reactivity was within normal limits in nearly every series of analysis. Two weeks following the last transfusion, the biuret linkages of both serum albumin and globulin fractions had returned to normal.

DISCUSSION

Considerable evidence has been presented in the literature to show that qualitative as well as quantitative changes occur in serum proteins in various diseases. Analysis of the data presented in Table I has shown that very significant changes occur in the relationship between biuret linkages and total nitrogen of serum globulin during liver disease. A difference in the distribution of serum globulin fractions in normal and cirrhotic individuals has been shown by electrophoretic and immunologic means.[14] There is much evidence that fibrinogen production is wholly dependent upon liver function. The role of the liver in the production of albumin and globulin is not as well established. There is, however, clinical and experimental evidence that the organ is involved in the processes of albumin and globulin formation, especially the former. Whipple and co-workers[15, 16] noted a lag in the regeneration of serum proteins of Eck fistula dogs following acute plasma depletion. Hepatectomy experiments in various animals have added little significant evidence relating to the origin and

mode of formation of plasma albumin and globulin since such a drastic procedure gives rise to profound systemic and circulatory disturbances. Hypoproteinemic dogs[17] exhibited a decreased deamination in impaired livers.

A relative loss of biuret linkages of serum globulin together with a concurrent gain of biuret linkages of albumin in toxic thyroid disease may indicate that the thyroid has some influence on formation, loss, or exchange of biuret linkages in serum proteins. Since no significant increase in biuret linkages of serum albumin occurred in liver disease, these alterations of serum albumin in thyroid disease cannot be explained as a result of secondary liver disease.

The tendency for overproduction of biuret linkages of both albumin and globulin in some anemias studied (Table I) may indicate a disturbance of a part of the normal function of blood cells in the formation of serum proteins. Hyperproteinemia observed in myeloid leucemia may indicate that serum globulin has its origin in bone marrow.[18] Since plasma proteins are within normal limits in aplastic anemia, more evidence is necessary to establish the proper function of marrow cells in protein formation.[19] However, the globulin:albumin ratios for aplastic anemia are above normal.[20] In No. 1 (Table II), during sickle cell anemia, an enormous overproduction of biuret linkages equal to 1 gram per cent protein N in the globulin fraction occurred. In multiple myeloma, No. 3 (Table II), a large increase in biuret linkages in the serum globulin occurred in the early acute stage of the disease when there was probably an overstimulation of the red marrow. Two to three months later, a decrease of biuret linkages in serum globulin was observed. This probaly was caused by the impaired activity of either the red marrow or liver. The production of a serum protein greatly altered in normal structure may be an important factor in the origin of the abnormal Bence Jones protein of multiple myeloma. Loss of biuret linkages of the globulin fraction in blood serum during chronic febrile disease similar to that observed in liver disease probably results from secondary liver damage caused by a fever of long duration. From a study[20] of the electrophoretic patterns of a series of normal and pathologic human sera, it was found that the serum α-globulin:albumin ratios were high in febrile patients, the average value being over twice the normal. This was particularly marked in pneumonia.[21, 22] Polarographic study[23, 24] of blood sera in experimental pneumonia (Type 1) in dogs has shown a decrease in wave heights in the denaturation curves which represented some change in serum (peptone fraction), probably a decrease in protein sulfhydral and disulfide groups, i.e. a decrease in cystine in peptide linkage. In acute infections, a protein has been found in the albumin fraction which gave a flocculation reaction with C polysaccharide of pneumococcus.[25] Electrophoresis has shown a decreasing albumin and increasing globulin in tuberculosis with the appearance of a new X component.[26]

The reversal of biuret and total nitrogen ratio in serum protein (Table II) over a comparatively short period of time in some diseases may indicate some relationship between albumin and globulin as to their interdependence, common origin, and mode of formation. It will be noted from the diagrammatic representation (Fig. 1) of the simultaneous gain and loss of biuret linkages in serum

albumin and globulin of Nos. 1, 2, and 3 presented in Table II that there is a definite relationship between albumin and globulin as to their relative gain or loss of biuret linkages. In No. 2, especially, it is apparent that a gain or loss of biuret linkages in albumin is matched by an equal change in the opposite direction in the globulin. In all three cases an approach to normal in biuret

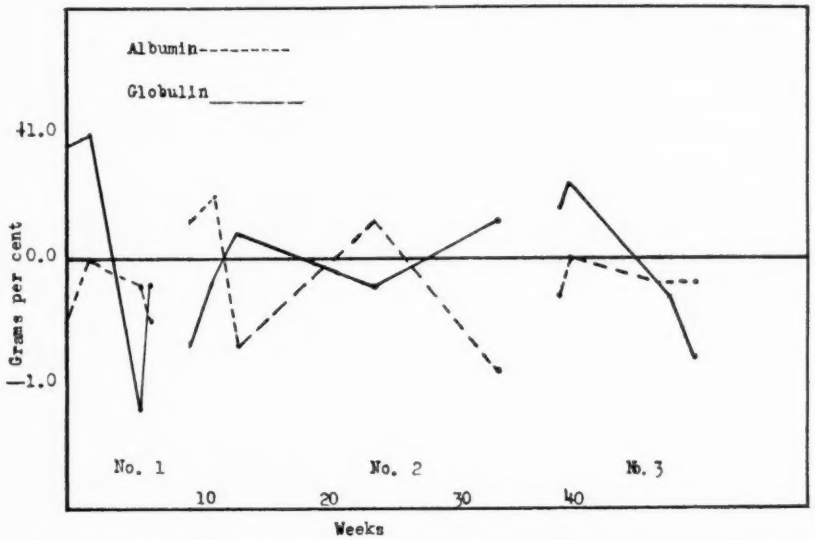

Fig. 1.—Diagrammatic representation of simultaneous gain and loss (grams per cent N) of biuret linkages in serum albumin and globulin (data from Table II).

linkages in both albumin and globulin occurs at the same time. In studies of protein metabolism by the isotope method,[27] synthesis and degradation were found to be a balanced biochemical process which requires no distinction between exogenous and endogenous cellular metabolism. Labile protein reserves have been demonstrated[28] and may pass readily from cell to plasma, and plasma to cell.[29] A depletion of these reserves lowered resistance to infection[30] and increased susceptibility to liver injury.[31, 32] These reserve protein stores have not been identified chemically.[33] The interchange of biuret linkages between albumin and globulin indicates the possibility of a normal exchange of biuret N between albumin and globulin, and/or they have a common origin in a labile protein reserve.

In most of the diseases studied it appears that the biuret linkages of the globulin are more labile and undergo wider variations than those of albumin. Liver, typhoid, and thyroid diseases show a significant loss of biuret linkages in serum globulin. In these diseases the biuret exchange of a precursor type of albumin with globulin may be arrested. This also may be related to the greater molecular size of the globulin molecule.

It is interesting to note the changes in biuret linkages of serum proteins in a case of nephrotic syndrome (23 to 32 Gm. protein loss per twenty-four hours) (Table II) twenty-three to thirty hours after a transfusion of 300 to

450 ml. of plasma. Total biuret linkages remain little changed while equally large losses in the albumin and gain in the globulin biuret linkages occur over a period of about four weeks. A response of this kind would certainly indicate a lack of normal utilization of the transfused proteins. This patient exhibited biuret linkages within normal limits in both albumin and globulin before and several days after the last transfusion. The gaining of biuret linkages in globulin at the expense of albumin may be an indication that albumin acts as an intermediary labile protein or that the mechanism for the formation of albumin is repressed as might be expected where the A/G ratio is extremely low. It is also interesting to note that the A/G ratios by biuret determination remained abnormally low and unchanged after transfusion, while by Kjeldahl determination the A/G ratios were normal in four instances.

The possible diagnostic value of the determination of the ratio of biuret N to Kjeldahl N in serum proteins is obvious, since changes in ratio of biuret N to total N of serum proteins in various diseases show definite patterns and in some diseases Kjeldahl determinations indicate normal serum proteins when their biuret linkages are abnormal.

Lack of agreement between determinations by the Kjeldahl and biuret methods may result from (1) a change in nitrogen concentration, (2) a change in number or reactivity of biuret linkages, (3) a change in response to salting out with sodium sulfate, or (4) a combination of (1), (2), and (3). These changes may be related to alterations in number, kind, or structural arrangement of amino acids in the protein molecule, or altered content of sugars, lipids, etc., forming part of the protein molecule. Of the three possibilities, (1) seems more likely to be the cause of the lack of agreement of the two analytic methods since the biuret linkages are so numerous and fundamental in protein structure that rupture or replacement of these bonds by other types would probably result in greater physical changes in plasma or serum proteins than those observed in the conditions studied. However, both (1) and (2) may be simultaneously operative or each may predominate under certain conditions. Peters and Van Slyke[34] mention the altered chromogenic power of serum proteins in disease, in reference to the use of tyrosine as a standard in the Greenberg[35] serum protein method. The possibility that polypeptides, amino acids, or other biuret reacting compounds were causing the differences observed was investigated in several cases where unusual differences between the biuret and Kjeldahl methods were noted. The concentration of these compounds was never high enough in the plasma or serum to have any appreciable effect on the biuret reaction.

Incomplete digestion of some amino acids of proteins have been reported,[36] but errors of this kind could not have occurred, since great care was exercised in all of our Kjeldahl determinations. The Kjeldahl digestion technique used in this investigation agreed well with the method recommended in an exhaustive experimental survey of Kjeldahl methods.[37] Our Kjeldahl-biuret analyses are not in agreement with a report[38] of significant differences of protein determinations on normal human serum made by the Kjeldahl and Kingsley biuret methods. However, analyses for only four normal serums and one cirrhotic serum were reported.

SUMMARY

1. In various diseases, differences were observed in serum albumin and globulin determinations by the biuret and total N methods. In Laënnec's cirrhosis, Banti's syndrome, biliary cirrhosis, hepatomegaly due to other causes, thyrotoxicosis, and typhoid fever, the principal change is loss of biuret linkages by the globulin.

2. In certain anemias an increase of biuret linkages in both albumin and globulin is the rule, although a rapid reciprocal change of biuret reactivity can take place in the protein fractions in some instances.

3. In general it may be said that primary hepatic disease, or secondary liver damage resulting from impairment in circulation, malnutrition, fevers of long duration, severe infections, or tissue damage, will cause changes in the normal relationship between total N and biuret reactivity of the serum proteins.

4. Theoretical implications derived from experimental clinical data as to the chemistry of certain phases of serum protein formation or degeneration as well as the actual chemical change responsible for abnormal biuret N and total N ratios have been presented.

REFERENCES

1. Kingsley, G. R.: Differences Between Biuret and Kjeldahl Methods for Serum Protein Determinations in Chronic Liver Disease, J. Biol. Chem. **140**: lxix, 1941.
2. Kingsley, G. R., and Terzian, L. A.: Studies of the Differences Between Biuret and Kjeldahl Determinations of Serum Proteins. I. Experimental Peritonitis, J. Lab. & Clin. Med. **34**: 1175, 1949.
3. Kingsley, G. R., and Behrend, A. A.: Studies of the Differences Between Biuret and Kjeldahl Determinations of Serum Proteins. II. Effect of Occlusion of the Hepatic Artery and Ligation of the Gastroduodenal Artery on Serum Proteins, J. Lab. & Clin. Med. **34**: 1178, 1949.
4. Kingsley, G. R.: Direct Biuret Method for Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, J. Lab. & Clin. Med. **27**: 840, 1942.
5. Kingsley, G. R.: The Determination of Serum Total Protein, Albumin and Globulin by the Biuret Reaction, J. Biol. Chem. **131**: 197, 1939.
6. Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin, J. Biol. Chem. **133**: 731, 1940.
7. Myers, W. K., and Keefer, C. S.: Relation of Plasma Proteins to Ascites and Edema in Cirrhosis of the Liver, Arch. Int. Med. **55**: 359, 1935.
8. Snell, A. M.: Changes in Proteins of Blood in Hepatic Disease, Proc. Staff Meet., Mayo Clin. **10**: 489, 1935.
9. Foley, E. F., Keeton, R. W., Kendrick, A. B., and Darling, D.: Alterations in Serum Protein as an Index of Hepatic Failure, Arch. Int. Med. **60**: 64, 1937.
10. Israel, H. L., and Reinhold, J. G.: Detection of Cirrhosis and Other Diseases of the Liver by Laboratory Tests, J. Lab. & Clin. Med. **23**: 588, 1938.
11. Gottardo, P., and Winters, W. L.: Portal Cirrhosis. Correlation of Clinical, Laboratory, Peritoneoscopic and Autopsy Findings, Am. J. M. Sc. **204**: 205, 1942.
12. Levin, L., and Leathem, J. H.: Relation of Pituitary Thyroid and Adrenal Glands to Maintenance of Normal Serum Albumin and Globulin Levels, Am. J. Physiol. **136**: 306, 1942.
13. Levin, L., Leathem, J. H., and Crafts, R. C.: Effects of Adrenalectomy and Replacement Therapy on Serum Protein Levels of Cat, Am. J. Physiol. **136**: 776, 1942.
14. Kendall, F. E.: Studies on Serum Proteins; Identification of Single Serum Globulin by Immunological Means. Its Distribution in Sera of Normal Individuals and of Patients With Cirrhosis of Liver and With Chronic Glomerulonephritis, J. Clin. Investigation **16**: 921, 1937.
15. Kerr, W. J., Hurwitz, S. H., and Whipple, G. H.: Regeneration of Blood Proteins, Am. J. Physiol. **47**: 379, 1918.
16. Knutti, R. E., Erickson, C. C., Madden, S. C., Rekers, P. E., and Whipple, G. H.: Liver Function and Blood Plasma Protein Formation; Normal and Eck Fistula Dogs, J. Exper. Med. **65**: 455, 1937.

17. Goettsch, E., Lyttle, J. D., Grim, W. M., and Dunbar, P.: Amino Acid Studies; Plasma Amino Acid Retention in Hypoproteinemic Dog as Evidence of Impaired Liver Function, J. Biol. Chem. **144**: 121, 1942.
18. Magnus-Levy, A.: Ueber krystallisiertes und amorphes Bence Jones-Eiweiss; multiple myelome, Ztschr. f. physiol. Chem. **243**: 173, 1936.
19. Bennhold, H., Kylin, E., and Rusznyák, S., editors: Die Eiweisskörper des Blutplasmas, Dresden, 1938, Theodor Steinkopff, p. 41.
20. Longsworth, L. G., Shedlovsky, T., and Mac Innes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, J. Exper. Med. **70**: 399, 1939.
21. Blix, G.: Quantitative Bestimmung von elektrophoretisch getrennten Serumglobulinen, Ztschr. f. d. ges. exper. Med. **105**: 595, 1939.
22. Leutscher, J. A.: Serum Albumin. II. Identification of More Than One Albumin in Horse and Human Serum by Electrophoretic Mobility in Acid Solution, J. Am. Chem. Soc. **61**: 2888, 1939.
23. Crossley, M. L., Kienle, R. H., Vassel, B., and Christopher, G. L.: Chemistry of Infectious Diseases; Polarographic Study of Blood Sera in Experimental Pneumonia in Dogs, J. Lab. & Clin. Med. **26**: 1500, 1941.
24. Crossley, M. L., Kienle, R. H., Vassel, B., and Christopher, G. L.: Chemistry of Infectious Diseases; Cystine Content of Hydrolysates of Blood Sera in Experimental Pneumonia in Dogs, J. Lab. & Clin. Med. **26**: 1635, 1941.
25. Abernethy, J. T., and Avery, O. T.: Occurrence During Acute Infections of Protein Not Normally Present in Blood; Distribution of Reactive Protein in Patients Sera and Effect of Calcium on Flocculation Reaction With C Polysaccharide of Pneumococcus, J. Exper. Med. **73**: 173, 1941.
26. Seibert, F. B., and Nelson, J. W.: Electrophoretic Identification of Antibody to Tuberculin Protein, Proc. Soc. Exper. Biol. & Med. **49**: 77, 1942.
27. Schoenheimer, R., and Rittenberg, D.: Study of Intermediary Metabolism of Animals With Aid of Isotopes, Physiol. Rev. **20**: 218, 1940.
28. Whipple, G. H.: Protein Production and Exchange in Body Including Hemoglobin, Plasma Protein and Cell Protein, Am. J. M. Sc. **196**: 609, 1938.
29. Madden, S. C., Turner, A. P., Rowe, A. P., and Whipple, G. H.: Blood Plasma Protein Production as Influenced by Various Degrees of Hypoproteinemia and by Amino Acids, J. Exper. Med. **73**: 571, 1941.
30. McNaught, J. B., Scott, V. C., Woods, F. M., and Whipple, G. H.: Blood Plasma Protein Regeneration Controlled by Diet: Effects of Plant Proteins Compared With Animal Proteins; the Influence of Fasting and Infection, J. Exper. Med. **63**: 277, 1936.
31. Miller, L. L., and Whipple, G. H.: Chloroform Liver Injury Increase as Protein Stores Decrease; Studies in Nitrogen Metabolism in These Dogs, Am. J. M. Sc. **199**: 204, 1940.
32. Messinger, W. J., and Hawkins, W. B.: Arsphenamine Liver Injury Modified by Diet. Protein and Carbohydrate Protective, But Fat Injurious, Am. J. M. Sc. **199**: 216, 1940.
33. Luck, J. M.: Liver Proteins; Question of Protein Storage, J. Biol. Chem. **115**: 491, 1936.
34. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry: I. Interpretations, Baltimore, 1932, Williams & Wilkins Company, p. 693.
35. Greenberg, D. M.: Colorimetric Determination of Serum Proteins, J. Biol. Chem. **82**: 545, 1929.
36. Miller, L., and Houghton, J. A.: The Micro-Kjeldahl Determination of the Nitrogen Content of Amino Acids and Proteins, J. Biol. Chem. **159**: 373, 1945.
37. Hiller, A., Plazin, J., and Van Slyke, D. D.: A Study of Conditions for Kjeldahl Determination of Nitrogen in Proteins, J. Biol. Chem. **176**: 1401, 1948.
38. Gornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751, 1949.

HEBERDEN'S NODES: THE RELATIONSHIP OF THE MENOPAUSE TO DEGENERATIVE JOINT DISEASE OF THE FINGERS

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HEBERDEN'S nodes are enlargements of the terminal interphalangeal joints of the fingers due to degenerative joint disease. Individual fingers are at times affected, especially in men, as a result of direct injury which produces a true traumatic arthritis. The deformities affecting multiple fingers on both hands, however, arise spontaneously without history of injury. The present study is confined to the latter type which is called idiopathic Heberden's nodes. Age is perhaps the most important single factor determining the appearance of this condition.[1] Heredity is also important, as is shown by vertical involvement in families in succeeding generations and horizontal involvement in siblings.[2] Women are affected much more commonly than men and, since Heberden's nodes are rare before the age of 50, their occurrence is thought to be related to the climacteric. The present study was made to test this supposition and to consider the mechanism by which the change of life influences the development of this deformity.

CLINICAL OBSERVATIONS

Observations were made on ninety-nine women with idiopathic Heberden's nodes who had passed the menopause and who gave a reliable history of the time of occurrence of the menopause and appearance of the nodes (Table I). The age of these women at the time of the onset of Heberden's nodes ranged from 33 to 65 years. The median age was 49.8 years; the average age was 48.8 ± 6.80 years. The age distribution at the time of onset of Heberden's nodes is shown in Fig. 1.

The age at the time of the menopause in this series of ninety-nine women ranged between 28 years, as the result of a pelvic operation, and 56 years. The median age was 49.7 years; the average age was 48.6 ± 5.25 years. The age distribution is shown in Fig. 2.

The history of the menopause in these ninety-nine women with Heberden's nodes was compared with that of a control series of ninety-six women (Fig. 3). The control series had been assembled and observed in a previous study under the same conditions and at the same time as the Heberden's nodes series. The control series included the sisters of forty-three women chosen from the wards of City Hospital simply because they did not have Heberden's nodes, a collection of families observed in connection with the studies on heredity of Heberden's nodes. In the control series the age at which the menopause occurred

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TABLE I. TIME RELATIONSHIP BETWEEN HEBERDEN'S NODES AND MENOPAUSE; NINETY-NINE CASES

CASE	PATIENT	AGE AT ONSET OF HEBERDEN'S NODES	AGE AT ONSET OF MENOPAUSE	INTERVAL (YR.)*
1	Co.	33	38	+ 5
2	Bl.	33	53	+20
3	Ba.	35	39	+ 4
4	Ra.	37	46	+ 9
5	Je.	38	28	-10
6	Go.	39	39	0
7	Ac.	39	50	+11
8	St.	40	46	+ 6
9	Br.	40	49	+ 9
10	Ra.	41	40	- 1
11	He.	41	41	0
12	Ra.	41	43	+ 2
13	Fr.	42	43	+ 1
14	Cr.	42	42	0
15	Ch.	42	40	- 2
16	Po.	42	46	+ 4
17	Ji.	42	48	+ 6
18	Ku.	42	50	+ 8
19	Pa.	43	43	0
20	Jo.	43	46	+ 3
21	Le.	43	52	+ 9
22	Ch.	44	38	- 6
23	Hi.	44	44	0
24	Hu.	44	40	- 4
25	Gr.	44	48	+ 4
26	Al.	45	47	+ 2
27	Mc.	45	45	0
28	Am.	45	46	+ 1
29	Br.	45	50	+ 5
30	He.	45	53	+ 8
31	Si.	45	53	+ 8
32	Ha.	46	47	+ 1
33	Mc.	46	47	+ 1
34	Ho.	46	56	+10
35	Li.	47	47	0
36	Wa.	47	48	+ 1
37	Fo.	47	50	+ 3
38	Ex.	47	50	+ 3
39	Du.	47	52	+ 5
40	Br.	48	33	-15
41	Wh.	48	48	0
42	Be.	48	49	+ 1
43	Cr.	48	50	+ 2
44	Fi.	48	50	+ 2
45	Pe.	48	55	+ 7
46	Ba.	49	35	-14
47	Sh.	49	50	+ 1
48	Sm.	49	51	+ 2
49	So.	49	51	+ 2
50	Do.	49	54	+ 5
51	Wa.	50	44	- 6
52	Wr.	50	45	- 5
53	Br.	50	48	- 2
54	Vo.	50	50	0
55	Ca.	50	50	0
56	Co.	50	52	+ 2
57	He.	50	52	+ 2
58	Ve.	50	52	+ 2
59	Th.	50	56	+ 6

TABLE I—CONT'D

CASE	PATIENT	AGE AT ONSET OF HEBERDEN'S NODES	AGE AT ONSET OF MENOPAUSE	INTERVAL (YR.)*
60	La.	51	54	+ 3
61	Br.	51	56	+ 5
62	Sm.	52	42	-10
63	Ha.	52	43	- 9
64	Bo.	52	47	- 5
65	La.	52	48	- 4
66	Sp.	52	49	- 3
67	St.	52	50	- 2
68	St.	52	55	+ 3
69	Br.	53	49	- 4
70	Sa.	53	50	- 3
71	Ca.	53	51	- 2
72	Un.	53	54	+ 1
73	Ha.	53	55	+ 2
74	Jo.	54	50	- 4
75	Mc.	54	51	- 3
76	Be.	54	52	- 2
77	Mc.	54	50	- 4
78	Fa.	55	50	- 5
79	Go.	55	48	- 7
80	Ha.	55	45	-10
81	Ca.	56	50	- 6
82	Le.	56	50	- 6
83	Mo.	57	51	- 6
84	Po.	57	52	- 5
85	Co.	57	53	- 4
86	Po.	57	55	- 2
87	El.	57	56	- 1
88	Kr.	58	47	-11
89	Mc.	58	50	- 8
90	Ba.	58	56	- 2
91	Yo.	59	49	-10
92	Sm.	59	52	- 7
93	Bi.	59	54	- 5
94	Me.	60	54	- 6
95	Pi.	61	52	- 9
96	Mi.	62	48	-14
97	He.	62	48	-14
98	Fl.	64	53	-11
99	Br.	65	51	-14

*The interval is given a plus sign (+) when the age at time of onset of the menopause was greater than the age at the onset of Heberden's nodes and a minus sign (-) when the reverse was the case.

ranged from 35 to 59 years. The median age was 49.1 years; the average age was 48.9 ± 4.90 years. The age distribution in the control series of women is shown in Fig. 3. Thus it is seen that the history of the menopause in ninety-nine women with Heberden's nodes paralleled closely that of ninety-six women who were used as controls. The history of the menopause in this group of ninety-nine women with Heberden's nodes was not considered to be remarkable.

The time relationship between the menopause and the onset of Heberden's nodes in each individual woman is shown in Fig. 4. The menopause preceded the onset of Heberden's nodes in forty-four instances and followed it in forty-five. Both events occurred in the same year in ten instances. The average difference in time between these two events regardless of which event occurred first was 4.9 ± 4.04 years. If the time interval between these events

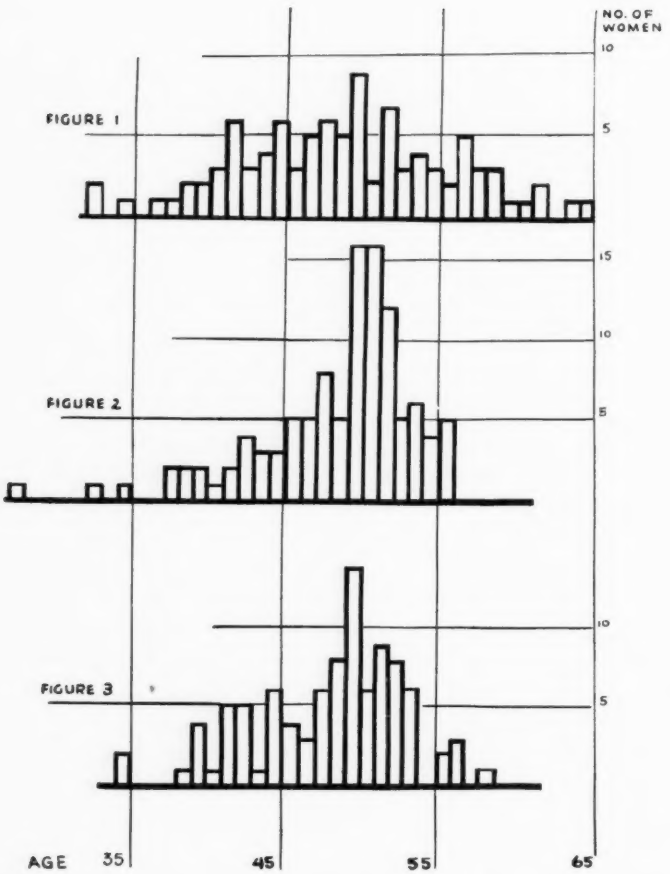

Fig. 1.—Age at the onset of Heberden's nodes in the ninety-nine women studied.

Fig. 2.—Age at the onset of the menopause in the ninety-nine women with Heberden's nodes.

Fig. 3.—Age at onset of the menopause in ninety-six women of the control series.

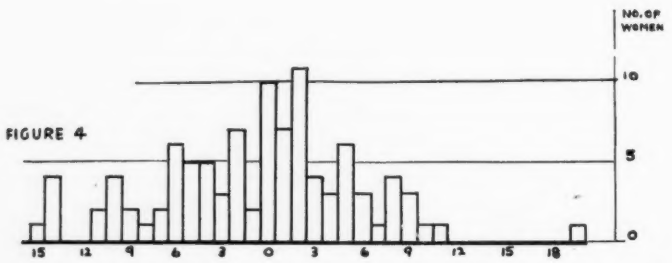

Fig. 4.—Time relationship between the age at onset of Heberden's nodes and the onset of the menopause in individual cases. Zero represents the age at onset of Heberden's nodes. Figures to the left of zero indicate the years before onset of Heberden's nodes at which the menopause occurred. Figures to the right of zero indicate the years after onset of Heberden's nodes at which the menopause occurred.

was computed always in the same direction, applying a positive sign to the number of years difference when the menopause followed the onset of Heberden's nodes and a negative sign when the menopause preceded the onset of Heberden's nodes, the average difference was -0.72 ± 6.33 years. The average difference practically disappeared and the two events seemed to be closely correlated in point of time. There was, however, a long time interval between these events in individual cases, the menopause occurring from twenty years after the onset of Heberden's nodes to fifteen years before. This is indicated by the large value for the standard deviation.

Despite the wide dispersion, close association of the menopause with the onset of Heberden's nodes was observed in many cases. In ten instances both events occurred in the same year. In forty-four instances, or nearly half of the series, they occurred within three years of each other. The time relationship of these two events, independent of the age at which they occurred, was then studied. As has been shown already, the average age of onset of Heberden's nodes in these ninety-nine women was 48.8 ± 6.8 and the average age for the menopause was 48.6 ± 5.2. When the group was divided into two equal parts on the basis of age, it was found that those women whose Heberden's nodes occurred early had an earlier onset of the menopause than those in whom the onset of the nodes was late. The average age of the menopause was 46.2 years in the fifty women having the earliest onset of Heberden's nodes at 33 to 49 years. The average age of the menopause was 50.5 years in the forty-nine women having later onset of Heberden's nodes at 50 to 65 years.

The degree of correlation between ages of onset and the menopause was calculated from grouped data by the usual product moment method for the determination of the coefficient of correlation (r). The value of r with its standard error is $+0.46 \pm 0.08$. By using the test recommended by Pearl,[3] this coefficient of correlation can be regarded as arising by chance from an uncorrelated population about once in a million times. The result leaves no doubt that the data show a highly significant statistical association between the ages of onset of Heberden's nodes and the menopause.

While a correlation coefficient of +0.46 is descriptive of the statistical association between the ages of onset of Heberden's nodes and the menopause, it gives no reliable indication of the causal factors involved. On the basis of certain assumptions, it is possible speculatively to draw a further conclusion by analogy with the interpretation of better known cases. It happens that the coefficient of correlation between stature of father and son is +0.46. Assuming that the number of elements causing the variability is the same in both instances and that they are normally distributed, the conclusion is drawn that about 46 per cent of the genes determining stature are common to parent and offspring of the same sex. It seems fair to make similar assumptions in the present instance. The data are consistent with this assumption, although they do not prove it. A number of factors obviously are involved in causing the variability in ages of onset of Heberden's nodes and of the menopause. The average age and the median age are about the same for both variables, indicating an approximately normal distribution for the causal factors involved.

Consequently, if the assumptions are justifiable, the conclusion is drawn that, on the average, 46 per cent of the total set of factors determining age of onset are common to Heberden's nodes and the menopause.

LITERATURE

Many observers have noted a close relationship between the menopause and Heberden's nodes. Heberden[4] himself made no mention of the menopause in his very short description of "digitorum nodi" and neither did Charcot.[5] Haygarth,[6] about the beginning of the nineteenth century, stated that nodosities of the joints are almost peculiar to women and generally begin about the period when the menses naturally cease. Duckworth,[7] who thought Heberden's nodes were related to gout, said, ". . . these may arise from the second to the fifth decade, are almost common perhaps as a gouty manifestation in women about or after the menopause." Monroe[8] stated that women display Heberden's nodes first about the menopausal period. Aschner[9] said he had seen Heberden's nodes often combined with the usual form of polyarthritis of the menopause. Cecil and Archer[10] noted Heberden's nodes in 74 per cent of the women in their series with menopausal arthritis. The *Primer on Rheumatism*[11] stated that Heberden's nodes often develop at the time of the menopause.

Besides the foregoing casual comments concerning the association of Heberden's nodes with the menopause, several students have presented statistical data to support the idea that the development of Heberden's nodes is related to ovarian dysfunction. Of forty-seven women studied by Pineles,[12] thirty-one noted the appearance of Heberden's nodes within a year of the last menstrual period. Four other women observed that their nodes occurred after the last menstrual period but while they were still having climacteric symptoms such as dizziness, nervousness, palpitation, hot flushes, and fleeting pains in various parts of the body. Four women under 45, though still menstruating regularly were having typical symptoms of the climacteric when their nodes began. Five women under the age of 40 stated that their nodes began shortly after pelvic operations which had resulted in cessation of the menses and menopausal symptoms. Only three of the forty-seven women developed Heberden's nodes independent of the menopause or menstrual dysfunction. Von Noorden[13] described Heberden's nodes and other manifestations of degenerative joint disease in women under 30. In all such instances he had noted abnormalities of the sexual phenomena such as amenorrhea, sterility, or atrophic genitalia. Albright and co-workers[14] described a 37-year-old woman with ovarian agenesis who had well-developed Heberden's nodes.

The clinical data seem to indicate a strong correlation between the climacteric and the development of Heberden's nodes. Since these deformities represent a particular manifestation of degenerative joint disease, it seemed pertinent to search the literature for evidence of correlation between the climacteric and this much broader and more general disease entity. The menopause is thought by some authors to cause or influence the onset of rheumatoid arthritis, by others to favor the development of osteoarthritis, while a third group believe its influence is limited to the production of arthralgia.[15] Hall,[16] studying seventy-one women who had been ovariectomized, found most of them suffering from symptoms of arthritis beginning a few weeks after operation and often suggesting true rheumatoid arthritis. Eighteen of his patients had symptoms of either true rheumatoid arthritis or osteoarthritis. He thought fifty-three of his patients had only arthralgia because no true joint disease developed.

The concept of arthropathia ovaripriva was discussed by Menge.[17] He observed a series of women having joint disturbances arising soon after inactivation of the ovaries by radiation. Complaints most often concerned the knees but frequently included the shoulder and occasionally the fingers. Involvement was most often bilateral. Crepitus on joint motion was frequently noted. Pain in some patients was slight but occasionally was severe enough to interfere with function. Menge was definitely of the opinion that the syndrome was due to ovarian failure, the same as occurred in spontaneous menopause.

Arthritis of the menopause was described by Cecil and Archer[10] as "a chronic polyarthritis of obese middle-aged women occurring at or just after the menopause and characterized by persistent stiffness and pain in the joints affected." Slight crepitation of the

knees was noted but limitation of motion or objective signs of joint disease were rare. Fisher[18] stated, "Disordered endocrine function plays an important part in many of the more chronic forms of arthritis occurring after the meridian of life. The type of arthritis that immediately springs to mind is the chronic osteoarthritis often seen in the knees of women, the onset of which corresponds with the menopause." The *Primer on Rheumatism*[11] stated that the menopause seems to be an exciting cause of hypertrophic arthritis. Jones[19] thought that osteoarthritis of the knees was most commonly met with in women at the menopause. Thomson and Gordon[20] thought climacteric arthritis was essentially the same as chronic synovitis which may eventually develop into typical osteoarthritis. Fox and Van Breemen[21] recognized menopausal arthritis as a generally accepted clinical entity. Radiographs show no change in bone and cartilage during the incipient stage which may be very long. Later the appearance resembles that of osteoarthritis. Steinbrocker[22] states that so-called menopausal arthritis consists merely of arthralgia associated with vasospastic symptoms of ovarian hormonal deficiency and that the symptoms are not of articular origin. Rheumatic pain was found to occur as a symptom of the climacteric in 24 per cent of 1,197 women by the British Subcommittee of the Council of the Medical Women's Federation.[23]

DISCUSSION

The nature of the phenomena considered in this study is such that exact data are not always obtainable. The development of Heberden's nodes is a slow process and its beginning cannot be identified with certainty. The degree of development necessary for recognition depends upon the discernment of the patient as influenced by her temperament, her degree of introspection, and her experience in having seen the deformity in her mother, sister, or intimate friends. Identification of the date of onset always seems more definite and apparently more exact the more recent it has been. It is almost equally convincing to hear that in one instance both events occurred about twenty years previously, the fingers not enlarging until one year after the last menstrual period. An uncertainty is suggested by a statement that the last menstrual period was six years previously and that the fingers began to enlarge about twenty years ago. To discard such data because they seem inexact introduces a different and more significant error in the study by eliminating those cases in which onset and menopause were not closely associated in point of time. The mere fact that onset and menopause were separated by many years and that both events were remote casts doubt only upon the exact time relationship of the two events and not on the fundamental fact that their occurrence was separated by a wide lapse of time. In several instances patients were observed personally for years while these events were occurring. In Case 7 the patient continued to menstruate for six years after finger enlargement had first been noted. Her mother, Case 96, was seen before her fingers enlarged. A series of photographs of her hand made for another purpose showed the onset of nodes at about 62 years of age, although her menopause occurred at 48 years. Cases were excluded only when the patients were definitely uncertain of the dates of the events under discussion.

Although the date of the last menstrual period was ascertained with reasonable certainty, the date of the onset or the duration of the climacteric was not easily identified. Removal of the uterus may stop menses without interfering with ovarian function. In Case 5 the uterus was removed when the

patient was 28 and nodes began at the age of 38. There were no hot flushes or other symptoms of the climacteric. In Case 39 the patient noted that nodes began to appear when menses became irregular five years before the menopause. In Case 40 the menopause occurred when the patient was 33, after both ovaries and tubes were said to have been removed, but nodes did not begin until the age of 48. In Case 46 a hysterectomy was done at the age of 35 and hot flushes continued until examination shortly after Heberden's nodes appeared at the age of 49. If adjustments for such data were made they would intensify the positive association between the onset of Heberden's nodes and the climacteric. We have used the last menstrual period for our computations.

Heberden's nodes arising idiopathically without relation to trauma are a particular manifestation of degenerative joint disease. The exact cause of this type of degenerative joint disease is not known. The incidence in any segment of the population is definitely related to age, varying from zero in women under 20 and reaching 30 per cent in the eighth decade. Because the disease produces a permanent deformity and develops at widely different ages in individual instances, the incidence is bound to increase as age advances after the second decade.

A strong genetic factor has been demonstrated as shown by the fact that the mothers of affected women have the disease twice as often and the sisters three times as often as the population in general.[2] A study of pedigrees and of gene frequency analyses indicated that the condition depends upon a single autosomal factor which is sex influenced so that it is dominant in women and recessive in men. It was further determined that 30 per cent of the female population was genotypically conditioned to develop the deformity.[24] According to this theory, Heberden's nodes will develop only in individuals with the proper genetic constitution for the trait. It is obvious that every woman in the present study was genotypically susceptible and therefore alike in this respect. They were all at least heterozygous. It is possible that a few were homozygous but not more than two or three such are to be expected statistically. The homozygous women cannot be distinguished from the heterozygous by observation.

Idiopathic Heberden's nodes also occur in men, but the disease is much less common than in women. It develops only in men who are homozygous for the trait and can be expected in less than 3 per cent of the male population over the age of 50 years. The disease in men has been less thoroughly studied than it has been in women so that its characteristics are not so well known.

Of the manifold symptoms of the menopause, the majority are not the direct result of ovarian failure as such but are due to change in activity of the pituitary gland and to derangement of function of the autonomic nervous system. The menopausal syndrome is characterized by loss of normal vasomotor balance as indicated by hot flushes, headaches, dizziness, hypertension, and numbness and stiffness of the hands and arms during the night. Circulatory disturbances are important in the etiology of degenerative joint disease.

This was proved by Goldhaft, Wright, and Pemberton[25] who produced hypertrophic arthritis with spur formation in dogs by interfering with the blood supply to joints. Brogsitter[26] described histologic evidence of decreased blood supply in Heberden's nodes. He found the blood vessels in the bones and soft tissue of these lesions to be decreased in size and number. It is probable that menopausal arthritis is due in part at least to circulatory disturbances. These can lead to Heberden's nodes only in those women who are genotypically susceptible. The genes for Heberden's nodes are already present in the fertilized ovum but their characteristic effect does not show itself until relatively late in life. Thus it seems clear that a long series of causes intervenes before Heberden's nodes appear. Indeed to discover these causes constitutes a study in physiologic genetics. If it could be shown definitely that circulatory disturbances are a necessary condition for their appearance, this would be to that extent a beginning in understanding something of the physiologic action of the genes for Heberden's nodes.

SUMMARY AND CONCLUSIONS

In this series of ninety-nine women the age at onset of Heberden's nodes was compared with the age at the menopause and these phenomena were found to be related to each other. Both events occurred within one year of each other in ten instances and within three years of each other in half the cases. In the individual patients the appearance of Heberden's nodes occurred from twenty years before to fifteen years after the menopause. The coefficient of correlation was found to be +0.46, indicating odds less than one to one million that this degree of correlation would occur by chance alone. Even this high degree of correlation does not indicate that the climacteric has a causal relation to Heberden's nodes. It is more likely that both events have etiological factors in common. Both must be considered as manifestations of the aging process. Since Heberden's nodes occur only in people who are genotypically susceptible, the climacteric can be considered as only a contributory, though a very important, factor in their production. The principal effect of the climacteric on the production of Heberden's nodes may be due to alterations in the peripheral circulation. This is the beginning of an understanding of the physiologic action of the genes for Heberden's nodes.

REFERENCES

1. Stecher, R. M.: Heberden's Nodes: The Incidence of Hypertrophic Arthritis of the Fingers, New England J. Med. **222**: 300-308, 1940.
2. Stecher, R. M.: Heberden's Nodes: Heredity in Hypertrophic Arthritis of the Finger Joints, Am. J. M. Sc. **201**: 801-809, 1941.
3. Pearl, R.: Medical Biometry and Statistics, Philadelphia, 1940, W. B. Saunders Company, p. 420 and Table B, p. 473.
4. Heberden, W.: Commentaries on the History and Cure of Diseases, ed. 2, London, 1803, T. Payne, p. 148.
5. Charcot, J. M.: Clinical Lectures on Senile and Chronic Diseases, New Sydenham Society, London, 1881.
6. Haygarth, J.: A Clinical History of Diseases, Part 2, A Clinical History of the Nodosity of the Joints, London, 1805, Cadell & Davies.
7. Duckworth, J.: A Treatise on Gout, London, 1889, C. Griffin & Company.
8. Monroe, R. T.: Chronic Arthritis, London, 1939, Oxford University Press, p. 61.

9. Aschner, B.: Treatment of Arthritis and Rheumatism in General Practice; Particularly in Women, New York, 1946, Froben Press, p. 73.
10. Cecil, R. L., and Archer, B. H.: Arthritis of the Menopause; a Study of Fifty Cases, J. A. M. A. **84**: 75-79, 1925.
11. Primer on Rheumatism; Chronic Arthritis. American Committee for the Control of Rheumatism cooperating with Committee on Scientific Exhibit, American Medical Association, 1934.
12. Pineles, F.: Zur Pathogenese der Heberdenschen Knoten, Wien. klin. Wchnschr. **21**: 902-904, 1908.
13. von Noorden: Discussion: Ueber den Heberdenschen Knoten, by Dr. L. Wick, Wien. med. Wchnschr. **58**: 1795, 1908.
14. Albright, F., Smith, P. H., and Fraser, R.: A Syndrome Characterized by Primary Ovarian Insufficiency and Decreased Stature, Am. J. M. Sc. **204**: 625-648, 1942.
15. Hench, P. S., and others: Problem of Rheumatism and Arthritis; Review of American and English Literature for 1937, Ann. Int. Med. **12**: 1295-1374, 1939.
16. Hall, F. C.: Menopause Arthritis, Proc. Am. Rheu. Assn., J. A. M. A. **113**: 1061, 1939.
17. Menge, C.: Ueber Arthropathia ovaripriva, Zentralbl. f. Gynäk. **48**: 1617-1621, 1924.
18. Fisher, A. G. T.: Chronic (Non-tuberculous) Arthritis: Pathology and Principles of Modern Treatment, New York, 1929, The Macmillan Company, p. 149.
19. Jones, R. L.: Arthritis Deformans; Comprising Rheumatoid Arthritis, Osteoarthritis and Spondylitis Deformans, New York, 1909, William Wood & Company, p. 251.
20. Thomson, F. G., and Gordon, R. G.: Chronic Rheumatic Diseases, London, 1926, Humphrey Milford.
21. Fox, R. F., and Van Breemen, J.: Chronic Rheumatism; Causation and Treatment, London, 1934, J. & A. Churchill, Ltd.
22. Steinbrocker, O.: Arthritis in Modern Practice; the Diagnosis and Management of Rheumatic and Allied Conditions, Philadelphia, 1941, W. B. Saunders Company.
23. Report of the Subcommittee of the Council of Medical Women's Federation, Lancet **1**: 106, 1933.
24. Stecher, R. M., and Hersh, A. H.: Heberden's Nodes; Mechanism of Inheritance in Hypertrophic Arthritis of the Finger Joints, J. Clin. Investigation **23**: 699-704, 1944.
25. Goldhaft, A. D., Wright, L. M., and Pemberton, R.: Influence of Age in the Experimental Production of Hypertrophic Arthritis; Preliminary Report, Ann. Int. Med. **6**: 1591-1598, 1933.
26. Brogsitter, A. M.: Mikroskopische Befunde bei Heberdenschen Knoten, Verhandl. d. deutsch. Gesellsch. f. inn. Med., Kong. **40**: 640-642, 1928.

THE STATE OF COMPONENT A (PROTHROMBIN) IN HUMAN BLOOD; EVIDENCE THAT IT IS PARTLY FREE AND PARTLY IN AN INACTIVE OR PRECURSOR FORM

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THE simple concept expressed in the classical theory of Morawitz and of Fuld and Spiro that prothrombin is converted by thromboplastin (thrombokinase) and ionized calcium to thrombin has become untenable because of a series of recent findings. In 1943 Quick,[1] and the following year Owren,[2] discovered a new factor which is essential for the formation of thrombin. Recently many other investigators have contributed data to confirm and support these original observations, but as is to be expected in the development of a new subject, numerous conflicting interpretations have been offered. No successful attempt has been made to correlate the pertinent contributions, particularly of Munro and Munro,[3, 4, 5] Fantl and Nance,[6, 7, 8, 9] Seegers, Ware and associates,[10-18] and Alexander and co-workers.[19, 20, 21] Recently we have obtained experimental findings which appear to bridge the conflicting and seemingly hostile views on prothrombin conversion currently expressed.

In the study made in 1943,[1] it was observed that when stored oxalated human plasma was mixed with an equal volume of dog plasma in which prothrombin had been reduced by Dicumarol or adsorbed with aluminum hydroxide, the resulting mixture had a prothrombin time of 9 to 10 seconds, which was shorter than that of fresh plasma. Three years later,[22] it was found that human citrated plasma (1 vol. of 0.1M sodium citrate to 9 vol. of blood) often showed a reduction of the normal prothrombin time of 12 seconds to as low as 9 seconds after twenty-four hours of storage. No explanation for these findings could be offered until 1948[23] when it was recognized that two changes were concurrently taking place in stored plasma, namely a decrease in the labile factor and an increase in some other factor which augmented prothrombin activity. Due particularly to the development of an assay method for component A,[24] and also to the discovery of the effect of silicone-coated containers on prothrombin activity during storage, it has been possible to obtain data which offer not only an explanation for the increased prothrombin activity of aged plasma, but also a new concept concerning the state of prothrombin in blood.

Since the terminology of the prothrombin factors has become increasingly more complex, it is essential that the principal terms employed in this study be defined. The labile factor is a constituent of both plasma and serum. It loses its activity when heated to 58° C. and is diminished in stored plasma because of oxidation. Removal of ionized calcium increases the speed of inactivation. Little, if any, of the labile factor is adsorbed by the amount of tricalcium

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phosphate which completely removes component A. It is not reduced either in Dicumarol poisoning or in vitamin K deficiency. Rabbit blood contains approximately fifty times as much labile factor as human plasma.[23]

Component A of prothrombin probably corresponds to the classical prothrombin. It is inactivated at 58° C., but does not diminish during storage. It is completely adsorbed by tricalcium phosphate and can be quantitatively recovered by elution with sodium citrate. Component A is diminished in Dicumarol poisoning and in avitaminosis K. Evidence has been presented to show that this component combines with the citrate ion, thereby losing activity and becoming no longer adsorbable by tricalcium phosphate.[25]

METHODS AND MATERIAL

The reagents and procedures employed were essentially the same as those described in a recent paper.[24] Silicone-coated glassware was prepared by means of Dri-Film 9987 (General Electric), using the technique described by Jaques and associates.[26] For high speed centrifugation, an angle centrifuge which had a maximum revolutions per minute of 4,000 was employed. For slow speed the revolutions per minute were 800 to 1,000 in an ordinary centrifuge.

The method of isolating component A by adsorption with tricalcium phosphate and elution with sodium citrate, which we recently described,[24] concentrates the active factor ten times. In its assay, therefore, 1 vol. was diluted with 9 vol. of calcium phosphate treated plasma. Rabbit plasma was used because it has an exceedingly high concentration of labile factor. The concentrate of component A is called eluate in this paper.

RESULTS

The Effect of Varying the Concentration of the Labile Factor on the Prothrombin Time When Component A is Kept Constant.—In a recent paper[24] it was shown that component A can be completely removed from oxalated plasma by an amount of tricalcium phosphate which takes out no detectable quantity of the labile factor. It was further shown that component A can be almost quantitatively recovered by elution with sodium citrate, and that this eluate when added to calcium phosphate plasma restores prothrombin activity provided sufficient labile factor is present. Since rabbit plasma contains fifty times and dog plasma ten times more labile factor than fresh normal human plasma and stored human plasma contains only a very small amount, treatment of these plasmas with tricalcium phosphate will yield testing media containing a wide range of concentrations of the labile factor. Such a series of deprothrombinized plasmas were employed to test the eluates from dog and human plasma. In the experiment, recorded in Fig. 1, 1 vol. of eluate was mixed with 9 vol. of deprothrombinized plasma. It will be observed that when the labile factor was low the prothrombin time was prolonged, and as the labile factor was increased, the prothrombin time decreased and reached a fixed minimal value. Dog eluate which contains a higher concentration of component A required more labile factor to attain a fixed minimal prothrombin time. If the labile factor were acting catalytically, its activity should continue as its concentration increases.

These results therefore suggest that the reaction of the labile factor and component A is stoechiometric. We have further found (unpublished data) that

when plasma deficient in labile factor clots, very little component A is consumed even when thromboplastin is in excess and the calcium concentration is optimal; but as the labile factor is progressively increased, the utilization of component A rises in proportion. Since these results support the view that the reaction between thromboplastin, component A, and labile factor is stoechio-

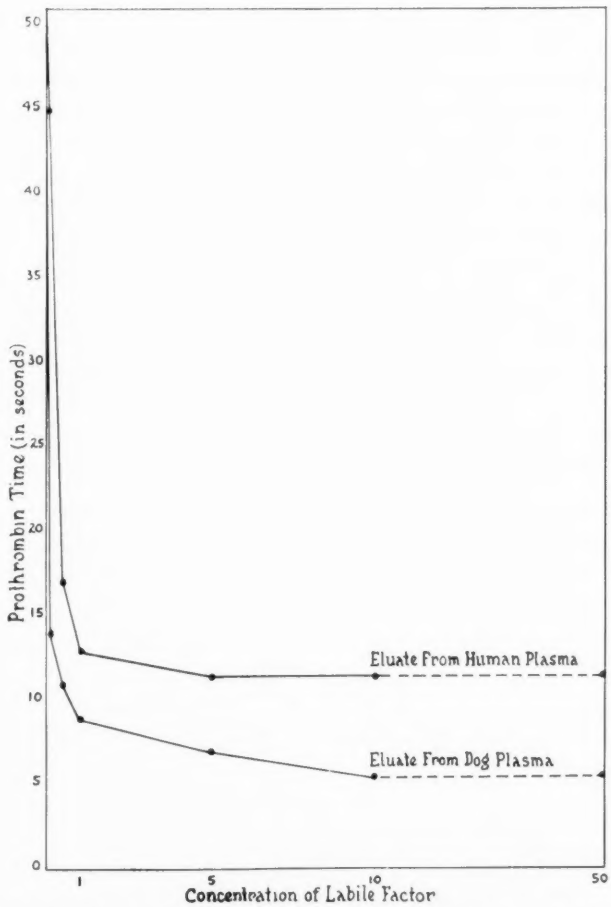

Fig. 1.—The influence of the concentration of the labile factor on the prothrombin time. The concentration designated as 50 was obtained by treating fresh oxalated rabbit plasma with tricalcium phosphate; 10 was dog plasma; 5, a mixture of dog and stored human plasma; and 1, fresh human plasma. One volume of eluate was mixed with 9 volumes of the plasma treated with tricalcium phosphate.

metric, one should question the correctness of calling the latter an accelerator. It is interesting to note that when Ware, Guest, and Seegers[11] corrected the two-stage method by adding an optimum amount of labile factor, they increased their yield of thrombin over 50 per cent. It can be postulated that at least three agents interact to form thrombin and that this reaction follows the law of mass action. The equation can be expressed as follows:

$$\text{A (component A)} + \text{L.F. (labile factor)} + \text{Tbp. (thromboplastin)} + \text{Ca (bound)} = \text{T (thrombin)}$$

At present there is insufficient data to determine whether all three agents interact simultaneously or whether two react to form a product which enters into a reaction with the third to form thrombin.

Evidence That Component A in Circulating Blood Exists Partly Free or Active and Partly in an Inactive State.—As already stated, when oxalated human plasma is stored in glass its prothrombin activity is markedly increased. Due to the fact that the labile factor is diminished on storage, this augmentation in prothrombin potency is masked and becomes demonstrable only by the addition of the labile factor. Interestingly, this increase in prothrombin activity does not occur or is greatly delayed if the plasma is stored in a silicone-coated container. It should be mentioned that Alexander and associates[21] observed that when blood clotted in silicone apparatus, the prothrombin activity of the serum was abnormally high, and the factor which they call serum prothrombin conversion accelerator was decreased.

The pertinent facts are brought out by the following experiment. Blood was obtained from a normal subject. A silicone-coated syringe chilled in an ice bath was employed. The blood was immediately oxalated, transferred to cold silicone-coated tubes, and centrifuged. Part of the plasma was put in Pyrex test tubes and the remainder in silicone-coated tubes. The amount of plasma in each tube was the same, and all were stored in an ordinary refrigerator. The results are summarized in Table I.

TABLE I. THE EFFECT OF TWENTY-FOUR HOUR STORAGE OF OXALATED HUMAN PLASMA IN GLASS AND SILICONE-COATED CONTAINERS ON COMPONENT A OF PROTHROMBIN

Rabbit $Ca_3(PO_4)_2$ plasma (c.c.)	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Normal oxalated human plasma (c.c.)	0.10	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
	Prothrombin Time (Sec.)									
Fresh human plasma in silicone	12½	12	12	12	12	12½	13½	15	20	28
Stored human plasma in silicone	17	12½	12	12	12	13	14	16	19	26
Stored human plasma in glass	13	9	8	8	8	8½	9	10	12	15

Rabbit $Ca_3(PO_4)_2$ plasma	0.09 c.c.
Eluate of oxalated human plasma	0.01 c.c.
Prothrombin Time:	
Fresh plasma eluate in silicone	12 sec.*
Stored plasma eluate in silicone	12 sec.
Stored plasma eluate in glass	7 sec.

*The eluate transferred to glass and stored for twenty-four hours gave a value of 7 seconds.

It is clear that the prothrombin time of the fresh plasma (12 seconds) was normal and that it was not increased by the addition of calcium phosphate treated rabbit plasma which is rich in labile factor. An eluate prepared from this plasma when added to rabbit calcium phosphate plasma yielded a prothrombin time of 13 seconds, which approximated the expected result. After twenty-four hours, the plasma in silicone-coated tubes lost labile factor, for the prothrombin time increased to 17 seconds, but component A remained unchanged since on adding rabbit calcium phosphate plasma a normal prothrombin time of

12 seconds was obtained. The eluate of this plasma yielded a prothrombin time of 12 seconds. In marked contrast, the plasma stored in glass, which had a prothrombin time of 13 seconds, showed when mixed with rabbit calcium phosphate plasma, i.e., with excess labile factor, a prothrombin time of 8 seconds. The eluate from this plasma yielded a prothrombin time of 7 seconds. Significantly, the eluate stored twenty-four hours in glass likewise showed the same increase in prothrombin activity. Usually maximum increase in prothrombin activity of plasma stored in glass is attained in twenty-four hours.

These results can best be explained by postulating that component A exists in a free form which is responsible for the prothrombin time of fresh plasma, and in an inactive state which becomes activated by storage. This activation requires a rough surface such as glass, but neither calcium nor thrombin are essential. Preliminary studies indicate that this reaction is very likely also independent of platelets. Eventually the activation occurs also in silicone-coated containers. No studies so far have been made to determine in what form inactive component A occurs, i.e. whether it is combined or merely existing as an inactive precursor. Tricalcium phosphate adsorbs both forms which accounts for the fact that the eluate on storage increases in potency. The nature of the activation remains undetermined.

The Concentration of Active and Inactive Component A in Hemophilic Blood.—In the development of the prothrombin consumption test,[27] it was frequently observed that the prothrombin time of serum was considerably shorter than the prothrombin time of plasma, which in hemophilia as in healthy subjects is 12 seconds. The solution to this anomalous finding was obtained by a simple experiment. Blood was collected from a hemophilic subject whose Lee-White coagulation time was 60 minutes. The usual silicone technique was employed. Part of the plasma was obtained by high centrifugation, the remainder by slow centrifugation. The native plasma in aliquotes of 3 c.c. was transferred to glass and silicone-coated test tubes. The prothrombin times were determined at regular intervals.

It will be observed from the results in Table II that the prothrombin time became progressively shorter during the course of an hour in the glass tubes but remained unchanged in the silicone tubes. At the end of forty-five minutes, the prothrombin times of both the highly and slowly centrifuged plasmas were 8½ seconds. The eluate likewise, which in the original plasma yielded a prothrombin time of 12 seconds, decreased in one hour to 7 seconds for the two plasmas kept in glass test tubes. It is clear that hemophilic plasma contains a normal concentration of both free and inactive component A, and that the activation of the latter occurs promptly in glass. The fact that the reaction occurs as fast in platelet-poor as in platelet-rich plasma makes it appear that the platelets play no direct part.

One of us (A.J.Q.)[27] has presented evidence showing that the hemophilic defect is a lack of thromboplastinogen, as a result of which the utilization of prothrombin is extremely small. Since hemophilic blood contains a normal concentration of both component A and the labile factor, but only a trace of throm-

TABLE II. THE CONCENTRATION OF FREE AND TOTAL COMPONENT A IN HEMOPHILIC BLOOD; THE ACTIVATING EFFECT OF GLASS SURFACE

TIME (MIN.)	PROTHROMBIN TIME OF NATIVE HEMOPHILIC PLASMA (SEC.)			
	HIGHLY CENTRIFUGED		SLOWLY CENTRIFUGED	
	GLASS (1) (SEC.)	SILICONE (2) (SEC.)	GLASS (3) (SEC.)	SILICONE (4) (SEC.)
0	11	11	11	11
10	11	11	11	11
20	10	11	10	11
30	9½	11	9	11
45	8½*	11	8½	11
120		11		11

Rabbit $Ca_3(PO_4)_2$ plasma		0.09 c.c.
Eluate of hemophilic plasma		0.01 c.c.
Prothrombin Time:		
Eluate of plasma 1	Before incubation	11 sec.
	After 60 min.	7 sec.
Eluate of plasma 2	Before incubation	11 sec.
	After 120 min.	11 sec.
Eluate of plasma 3	Before incubation	11 sec.
	After 60 min.	7 sec.
Eluate of plasma 4	Before incubation	11 sec.
	After 120 min.	7 sec.

*Coagulation began.

boplastin, nearly all of the prothrombin complex remains unconsumed after coagulation. Therefore almost all of component A remains even though hemophilic blood clots, and in addition the inactive A becomes converted to the free state with the result that total free or active component A greatly exceeds the amount initially present at the time the blood was drawn.

The Effect of Removing Platelets on the Conversion of Inactive Component A.—It was early observed that the prothrombin consumption time in thrombocytopenia was, as in hemophilia, actually shorter than the prothrombin time of normal plasma.[27] The problem could now be studied more effectively. Blood was drawn in a chilled silicone syringe and transferred to silicone-coated test tubes immersed in ice. The tubes were centrifuged for thirty minutes at 4,000 r.p.m. in an angle centrifuge. Three cubic centimeters of the platelet-poor plasma were put in a silicone-coated test tube and an equal amount in a glass tube.

It will be observed from the results in Table III that the prothrombin time in the silicone tube remained unchanged during one hour, whereas it shortened to 8½ seconds in less than twenty minutes in the glass tube. An eluate prepared immediately from the native plasma gave a prothrombin time of 12 seconds, and a similar value was obtained from the plasma remaining one hour in a silicone-coated tube. The eluate from the plasma in the glass test tube yielded a prothrombin time of 8 seconds.

In seeking the reason for these abnormally short prothrombin times of platelet-poor plasma in a glass container, the most satisfactory explanation is found in Quick's theory that thromboplastinogen is activated by a platelet enzyme. Since the platelets have been removed almost completely, only a trace of the activating enzyme is available and therefore only a minute amount of

TABLE III. THE EFFECT OF GLASS AND SILICONE SURFACES ON THE PROTHROMBIN ACTIVITY (CHANGE IN CONCENTRATION OF FREE COMPONENT A) OF PLATELET-POOR NORMAL NATIVE HUMAN PLASMA

PROTHROMBIN TIME OF NATIVE HUMAN PLASMA		
TIME (MIN.)	GLASS (1) (SEC.)	SILICONE (2) (SEC.)
0	12	12
10	10	11½
20	8½*	11½
30		11½
60		11½
Eluate Tested by Mixing With 9 Parts of Rabbit $Ca_3(PO_4)_2$ Plasma		
Plasma 1	Before incubation	12 sec.
	After 20 min.	8 sec.
Plasma 2	Before incubation	12 sec.
	After 60 min.	12 sec.

*Coagulation began at 15 minutes.

thromboplastin is formed. As in hemophilia, only a small fraction of the labile factor and component A is consumed in the reaction with thromboplastin, and since additional free component A is derived from inactive component A, the total amount of the latter is more than twice that of the original plasma.

TABLE IV. THE EFFECT OF SURFACE (GLASS AND SILICONE) ON THE PROTHROMBIN CONSUMPTION TIME WHEN THROMBIN* IS ADDED TO PLATELET-POOR NATIVE HUMAN PLASMA

TIME OF INCUBATION (MIN.)	PROTHROMBIN CONSUMPTION TIME†	
	IN GLASS (SEC.)	IN SILICONE (SEC.)
0	12	12
15	8	11
30	8	11
75	8	11

*To 1 c.c. of native plasma, 0.01 c.c. of thrombin (Quick's full strength) was added.

†The prothrombin consumption test time was determined by the procedure previously described.[27]

In order to determine whether thrombin is the agent responsible for the conversion of inactive component A to the active form, native platelet-poor plasma was placed in silicone and in glass test tubes and thrombin added to both. As seen from the results in Table IV, there is no indication that thrombin caused any activation of inert component A in silicone. Whether it participated in the reaction occurring in glass is difficult to decide.

The Effect of Dicumarol on the Concentration of Free and Inactive Component A.—Only preliminary studies have been made. The findings indicate that both free and the inactive component A are reduced. Further studies are in progress.

The Relative Ratios of Free to Inactive Component A in Congenital Hypoprothrombinemias.—There are three known types of congenital hypoprothrombinemia.[28, 29, 30] In one the labile factor is diminished while in the other two this agent is normal but component A is diminished. In the first type according to Owren,[30] who has reported the only case so far known in which the labile factor (Factor V) was apparently diminished from birth, the conventional prothrombin is quantitatively normal.

In types II and III of congenital hypoprothrombinemia the labile factor is normal. Both types show a prolonged prothrombin time by the one-stage method, but curiously when 1 vol. of type II is added to an equal volume of type III, the resulting mixture has a prothrombin time which is nearly that of normal plasma.[29]

To explain these results, Quick[28] postulated that one type (II) lacked component A and that the second type (III) was deficient in a hypothetical component B, and that when the plasmas of the two types were mixed in equal volumes they mutually corrected each other. With the new evidence obtained in this study that only a part of the total component A is present in the active state, a better interpretation of these findings can be offered and means to differentiate types II and III can be formulated.

From the results in Table V, the basic difference between the two types of congenital hypoprothrombinemia becomes clearly evident. In type II there is a definite lack of both free and total component A; whereas in type III, the free is diminished but the total may be practically the same as that of normal plasma. When plasma of type II is mixed with type III, sufficient inactive component A of the latter plasma is converted to the active form to bring the prothrombin time nearly to normal.

TABLE V. THE CONCENTRATION OF FREE AND TOTAL COMPONENT A IN THE TWO TYPES OF CONGENITAL HYPOPROTHROMBINEMIA IN WHICH THE LABILE FACTOR IS NORMAL

Rabbit $Ca_3(PO_4)_2$ plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Congenital hypoprothrombinemia plasma (c.c.)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
	Prothrombin Time (Sec.)									
Type II, fresh plasma	20	19½	19½	20½	21	22	23	26	35	53
Type II, after 4 days of storage in glass	37	17½	17	16	16½	16½	18½	21	28	42
Type III, fresh plasma	16	15	14½	15	15	16	17½	18½	22½	30
Type III, after 5 days of storage in glass	30	10	9	8	8	8½	8¾	9	11	17

The eluates were tested by adding 0.01 c.c. to 0.09 c.c. of rabbit plasma treated with $Ca_3(PO_4)_2$

	Prothrombin Time
Eluate of fresh plasma, type II	17 sec.
Eluate of stored plasma, type II	16 sec.
Eluate of fresh plasma, type III	13 sec.
Eluate of stored plasma, type III	7 sec.

Apparently there is in plasma a regulating mechanism which determines the ratio of free to total component A. In normal human plasma, the ratio is such that a prothrombin time of 12 seconds is obtained with the one-stage method. When all of component A is converted to the free state as occurs in storage, the prothrombin time becomes 7 to 8 seconds. Similar values are obtained when type III plasma is stored in glass even though the prothrombin time of the fresh plasma may be elevated significantly. Clearly, in this form of hypoprothrombinemia, the concentration of free component A is fixed at a level lower than normal. The fixation of the free prothrombin level appears definitely to be established by heredity. The affected members of the family who have

been studied in our laboratory[28, 29, 31] have a prothrombin time of 16 seconds. A second family has recently been studied in which the prothrombin time of the mother and her two children is 14 seconds. None of these subjects has a bleeding tendency. It is likely, however, that if the level of the free component A falls below a critical level, a hemorrhagic diathesis will develop. The patient studied by Hagen and Watson[32] very probably has a type III hypoprothrombinemia, and she has a severe bleeding condition.* In rabbits and dogs nearly all of component A is in the free state, according to our preliminary studies.

DISCUSSION

The findings reported in this paper promise to offer for the first time means to correlate and harmonize the significant contributions of Bordet,[33, 34] Nolf,[35] and the more recent investigators. It will be recalled that Bordet concluded that both cytozyme (thromboplastin) and serozyme (prothrombin) were mother substances of thrombin. He believed that the platelets liberated cytozyme when they came in contact with a rough surface. He further postulated that the serozyme in circulating blood was in an inactive state which he named proserozyme. For its activation, calcium and a rough surface were required. He concluded that tricalcium phosphate completely removed serozyme and assumed that the resulting "phosphate plasma" contained only one remaining clotting factor, namely fibrinogen. Nolf,[35] on the contrary, postulated that serozyme contained two factors, thrombozyme adsorbable by tricalcium phosphate and thrombogen which remained in the "phosphate plasma." Nolf unfortunately failed to recognize the importance of platelets and in his earlier work looked upon thrombin not as the cause of clotting but as a consequence of the reaction.

In agreement with Bordet, the present findings confirm the existence of a proserozyme, but differ in that not all but only a part of the serozyme is in this inactive state. The activation of proserozyme, i.e., the conversion of inactive component A, requires a rough surface like glass, but apparently is not dependent upon calcium as Bordet assumed since the activation occurs in oxalated plasma. Bordet was not aware that phosphate plasma still contained a clotting factor other than fibrinogen. Undoubtedly the thrombogen of Nolf is identical with the labile factor.

In the development of this subject the establishment of the concept that the interaction of thromboplastin with the prothrombin complex is stoechiometric was of basic importance. Mertz, Seegers, and Smith[36] showed that the amount of thrombin obtained from a fixed amount of prothrombin increased proportionately to the quantity of thromboplastin added until the optimum was reached. Later, Quick[37] showed that calcium likewise acted stoechiometrically in this reaction. With the discovery of the labile factor, and the finding that it shortened the prothrombin time, a new period of confusion set in. Quick postulated its action was stoechiometric, whereas Owren[38] claimed that it functioned in forming a prothrombin-converting enzyme. Fantl and Nance,[7] who studied this factor, concluded that it increased the speed of the conversion of prothrombin and they named it prothrombin accelerator. Munro and Munro[5]

*Dr. Paul S. Hagen kindly supplied us with blood from this patient and it was found that she lacked both free and total prothrombin.

believed that prothrombin on storage became partly inactivated by an alteration of a labile group in the molecule and could again be reactivated, but they apparently did not consider that the activating agent was the labile factor. Seegers and associates, who are now inclined to agree[18] that the agent which they named Ac-globulin is the same as the labile factor, assumed that it increased the conversion rate of prothrombin. Murphy and Seegers[17] attempted to explain the discrepancies in prothrombin values of different species obtained by the one- and two-stage methods on the variations of the concentration of Ac-globulin.

As already mentioned, we have presented experimental findings which show that increasing the concentration of labile factor manifold did not decrease the prothrombin time.[24] Assuredly, the prothrombin time should be a sensitive test of the speed of conversion of prothrombin. In our studies, we learned that consistent results were obtainable only if silicone glassware was used. Since Bordet's work had been forgotten, it was generally assumed that a rough surface influenced only platelet disintegration, with the result that conversion of inactive component A to the active state which occurred was not recognized and was misinterpreted as a conversion of prothrombin to thrombin. The serum prothrombin conversion accelerator of Alexander and co-workers[20] is very probably concerned with the activation of component A rather than with the conversion of prothrombin to thrombin.

Many of the findings on Ac-globulin by Seegers and his associates are difficult to interpret because much of their material was collected in slaughter houses and generally in large containers. Under such conditions, considerable changes could occur even before the blood was decalcified, especially since sodium oxalate is relatively slow in its action.[24] Fahey, Ware, and Seegers[18] observed no difference in the influence of silicone and glass surfaces on the stability of Ac-globulin, which agrees with our observations. They failed to note however that a glass surface has a basic influence in converting inactive component A to the active state, because the methods of study which they employed did not distinguish between free and total prothrombin (component A).

A rational explanation for the differences in prothrombin determination by the one- and two-stage methods can now be offered. By the one-stage procedure, the prothrombin activity of dog blood is almost five times as great as that of human blood, whereas by the two-stage method, the prothrombin of dog blood is only slightly higher than in human blood. As already stated, the prothrombin time of fresh human plasma is 12 seconds, but the time of stored blood with the labile factor restored may be 7 to 8 seconds. The prothrombin time of dog plasma is 6 seconds. Since it can be assumed that in stored blood all the component A becomes activated, one can see that the prothrombin activity of dog plasma and of human plasma with all of its prothrombin activated is of the same order of magnitude. In carrying out the two-stage method, the conditions are such that all of the prothrombin (component A) is converted to the active form. Therefore the method measures only total component A. The one-stage method, on the contrary, measures only free or active component A. The fact that component A can be quantitatively adsorbed and eluted and then tested on a standardized deprothrombinized medium makes it possible to

check the results of the one-stage method. By this means it has been found that dog plasma contains over four times as much free component A as human plasma.[24]

The establishment that component A, or the classical prothrombin, exists in human blood both in the free and in the precursor state creates a new problem. How and when does the inactive prothrombin become available in physiologic coagulation? From test tube experiments it is clear that a rough surface is required, but it is still uncertain whether such a surface is available intracorporally. It still remains to be determined whether the precursor component A functions directly in coagulation, or whether it serves as a supply of free component A.

It is obvious that the terminology now in general use is cumbersome and in some instances inaccurate. We have adhered to the term labile factor since it is descriptive and noncommittal. We see no justification for calling it an accelerator. Eventually, when its action is better defined, a more suitable name may be found. In regard to component A, it appears fairly certain that it is or is closely related to the classical prothrombin, the serozyme of Bordet, and the thrombozyme of Nolf. If component A be named prothrombin, it becomes difficult to decide what its inactive or precursor compound should be called. One term which suggests itself is prothrombinogen. Since such a term in recent enzyme chemistry implies that in its activation cleavage occurs, it seems advisable to postpone action until such time when the mechanism has been elucidated. For the present, the neutral terms component A and pre-A seem best.

SUMMARY

1. From studies based on the prothrombin time of plasma and from data obtained with the adsorption and elution of component A, it is concluded that the formation of thrombin requires component A, the labile factor, thromboplastin, and combined calcium. This reaction appears to follow the law of mass action. There is no evidence that any of the factors are accelerators.

2. In fresh human plasma, component A (prothrombin) is present partly free and partly in an inactive or precursor state which corresponds to the proserozyme of Bordet. It requires a rough surface for its activation, but there is no evidence that calcium, thromboplastin, or thrombin are essential for its conversion to the active state. In oxalated human plasma, nearly all of component A is changed to the active form after twenty-four hours of storage if kept in contact with a rough surface such as glass.

3. In the coagulation of plasma in which there is a deficiency of available thromboplastin due either to a lack of thromboplastinogen as in hemophilia or to removal of platelets, the concentration of free component A is markedly increased because little of the original active form is consumed and additional active component A is produced from the inactive precursor.

4. Two types of congenital hypoprothrombinemia are known in which a deficiency of component A occurs. In one type a true deficiency of both free and total component A exists. In the second type, which is the more common and is hereditary, the concentration of total component A is normal, but the

amount of free or active is below normal. This suggests that a mechanism, still completely unknown, regulates the ratio of active to total component A.

5. The present work confirms the early conclusions of Bordet that serozyme or prothrombin may exist in an inactive or precursor state; but, unlike Bordet's concept that all exists as proserozyme, the present findings show that a large fraction of the total component A is in an active form. It appears probable that in a number of recent studies the activation of component A has been misinterpreted as an acceleration of the conversion of prothrombin to thrombin.

6. Since the procedure of the two-stage method for determining prothrombin is such that all of the component A is converted to the active form, this method determines total prothrombin (component A) but does not distinguish between the free and inactive form. The one-stage method, on the contrary, determines the free or active component A and, to determine the total, all of component A must first be converted to the active state.

REFERENCES

1. Quick, A. J.: On the Constitution of Prothrombin, Am. J. Physiol. **146**: 212, 1943.
2. Owren, P. A.: New Investigation on the Coagulation of the Blood, Arbok for det Norske Videnskopsakademi, 1944, p. 21.
3. Munro, F. L., Hart, E. R., Munro, M. P., and Walkling, A. A.: The Changes in Components A and B of Prothrombin in the Dog Following Hepatectomy, Am. J. Physiol. **145**: 206, 1945.
4. Munro, F. L., and Munro, M. P.: The Interaction of Prothrombin A and B, Am. J. Physiol. **149**: 95, 1947.
5. Munro, M. P., and Munro, F. L.: The Reversible Inactivation of Prothrombin: A Factor Responsible for Its Partial Reactivation, Am. J. Physiol. **150**: 409, 1947.
6. Fantl, P., and Nance, M. H.: Acceleration of Thrombin Formation by a Plasma Component, Nature **158**: 708, 1946.
7. Fantl, P., and Nance, M. H.: Activation of Prothrombin, Australian J. Sci. **9**: 117, 1946.
8. Fantl, P., and Nance, M. H.: The Influence of Storage on the Coagulation Factors of Human Plasma, Australian J. Exper. Biol. & M. Sc. **26**: 207, 1948.
9. Fantl, P., and Nance, M. H.: The Physiological Activation of Prothrombin, M. J. Australia **1**: 128, 1948.
10. Ware, A. G., Guest, M. M., and Seegers, W. H.: A Factor in Plasma Which Accelerates the Activation of Prothrombin, J. Biol. Chem. **169**: 231, 1947.
11. Ware, A. G., Guest, M. M., and Seegers, W. H.: Plasma Accelerator Factor and Purified Prothrombin Activation, Science **106**: 41, 1947.
12. Ware, A. G., Murphy, R. C., and Seegers, W. H.: The Function of Ac-Globulin in Blood Clotting, Science **106**: 618, 1947.
13. Murphy, R. C., Ware, A. G., and Seegers, W. H.: Plasma Ac-Globulin Activity, Am. J. Physiol. **151**: 338, 1947.
14. Ware, A. G., and Seegers, W. H.: Plasma Accelerator Globulin: Partial Purification, Quantitative Determination, and Properties, J. Biol. Chem. **172**: 699, 1948.
15. Ware, A. G., and Seegers, W. H.: Serum Ac-Globulin: Formation From Plasma Ac-Globulin; Role in Blood Coagulation; Partial Purification; Properties; and Quantitative Determination, Am. J. Physiol. **152**: 567, 1948.
16. Ware, A. G., and Seegers, W. H.: Studies on Prothrombin: Purification, Inactivation With Thrombin, and Activation With Thromboplastin and Calcium, J. Biol. Chem. **174**: 565, 1948.
17. Murphy, R. C., and Seegers, W. H.: Concentration of Prothrombin and Ac-Globulin in Various Species, Am. J. Physiol. **154**: 134, 1948.
18. Fahey, J. L., Ware, A. G., and Seegers, W. H.: Stability of Prothrombin and Ac-Globulin in Stored Human Plasma as Influenced by Conditions of Storage, Am. J. Physiol. **154**: 122, 1948.
19. Alexander, B., and DeVries, A.: Human Prothrombin: Quantitative Studies on the Plasma Labile Factor and the Restorative Effect of Normal, Hypofibrinogenemic and Hemophilic Plasma on the Prothrombin of Stored Plasma, J. Clin. Investigation **28**: 24, 1949.

20. Alexander, B., DeVries, A., Goldstein, R., and Landwehr, G.: A Prothrombin Conversion Accelerator in Serum, Science **109**: 545, 1949.
21. DeVries, A., Alexander, B., and Goldstein, R.: A Factor in Serum Which Accelerates the Conversion of Prothrombin. I. Its Determination and Some Physiologic and Biochemical Properties; II. Its Evolution With Special Reference to the Influence of Conditions Which Affect Blood Coagulation, Blood **4**: 246 and 739, 1949.
22. Quick, A. J.: Influence of Decalcification on the Determination of Prothrombin, Federation Proc. **5**: 150, 1946.
23. Quick, A. J., and Stefanini, M.: The Concentration of the Labile Factor of the Prothrombin Complex in Human, Dog, and Rabbit Blood. Its Significance in the Determination of Prothrombin Activity, J. Lab. & Clin. Med. **33**: 819, 1948.
24. Quick, A. J., and Stefanini, M.: The Concentration of Component A in Blood, Its Assay and Relation to the Labile Factor, J. Lab. & Clin. Med. **34**: 973, 1949.
25. Quick, A. J., and Stefanini, M.: The Chemical State of the Calcium Reacting in the Coagulation of Blood, J. Gen. Physiol. **32**: 191, 1948.
26. Jaques, L. B., Fidlar, E., Feldsted, E. T., and MacDonald, G. G.: Silicones and Blood Coagulation, Canad. M. A. J. **55**: 26, 1946.
27. Quick, A. J.: Studies on the Enigma of the Hemostatic Dysfunction of Hemophilia, Am. J. M. Sc. **214**: 272, 1947.
28. Quick, A. J.: Congenital Hypoprothrombinaemia and Pseudo-hypoprothrombinaemia, Lancet **2**: 379, 1947.
29. Quick, A. J.: Components of the Prothrombin Complex, Am. J. Physiol. **151**: 63, 1947.
30. Owren, P. A.: Parahaemophilia-Haemorrhagic Diathesis Due to the Absence of a Previously Unknown Clotting Factor, Lancet **1**: 446, 1947.
31. Quick, A. J.: Effect of Synthetic Vitamin K and of Quinine Sulfate on the Prothrombin Level, J. Lab. & Clin. Med. **31**: 79, 1946.
32. Hagen, P. S., and Watson, C. J.: Idiopathic (Familial) Hypoprothrombinemia, J. Lab. & Clin Med. **33**: 542, 1948.
33. Bordet, J.: Recherches sur la coagulation du sang. Serozyme et proserozyme, Soc. de Biol. **82**: 896, 1919.
34. Bordet, J.: The Theories of Blood Coagulation, Bull. Johns Hopkins Hosp. **32**: 213, 1921.
35. Nolf, P. A.: Le plasma phosphaté reactif de la coagulation du sang, Arch. internat. de pharmacodyn. et de thérap. **70**: 5, 1945.
36. Mertz, E. T., Seegers, W. H., and Smith, H. P.: Prothrombin, Thromboplastin and Thrombin: Quantitative Interrelationship, Proc. Soc. Exper. Biol. & Med. **42**: 604, 1938.
37. Quick, A. J.: Is the Action of Calcium in the Coagulation of Blood Stoichiometric or Catalytic? Science **106**: 591, 1947.
38. Owren, P. A.: Coagulation of Blood; Investigations on New Clotting Factor, Acta med. Scandinav., supp. **194**: 1, 1947.

FAILURE OF SENSITIZED SHEEP CELL AGGLUTINATION TO CLARIFY THE DIAGNOSIS OF RHEUMATIC DISEASE

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INTRODUCTION

EARLY joint pain in a patient often presents a difficult diagnostic problem. For this reason, the report by Rose, Ragan, Pearce, and Lipman[1] of a test that was diagnostic for active rheumatoid arthritis was received with much enthusiasm. However, much more study is indicated before the reliability of this test is established and before it can be placed in the hand of the clinician as a proved aid to the diagnosis of active rheumatoid arthritis.

Rose and co-workers[1] found that the sera of patients with active rheumatoid arthritis agglutinated sheep cells sensitized with rabbit antisheep cell serum in a much higher titer than they agglutinated plain sheep cells. In their series, the sera of twenty-seven patients with active rheumatoid arthritis were tested. Four of the sera had differential titers of 16 and the remainder had differential titers exceeding this level. Also reported in this series were sixteen cases of inactive rheumatoid arthritis, five of which had differential titers exceeding 16, while the remainder had differential titers ranging from zero to 16. Included in their report were twenty-nine patients without evidence of arthritis. Two of these patients had a differential titer of 16, while the remaining twenty-seven had a titer of eight or less.

Cases of rheumatoid spondylitis, rheumatic fever, arthritis other than rheumatoid arthritis and rheumatic fever, infectious mononucleosis, and Still's disease were included in their study, all but two demonstrating differential agglutination titers below 16. They concluded that a sixteen fold difference or more occurs almost exclusively with the sera of patients with rheumatoid arthritis, and that the magnitude of the difference in titer usually reflects the clinical activity of the disease.

It is the purpose of this paper to present our results with this procedure; to correlate, if possible, the results of this test with the activity of the disease; and to determine how much promise it might hold for the clinician in the differential diagnosis of the arthritides.

METHODS AND MATERIALS

In our initial series we used the method of Rose and associates[1] as follows. The serum of patients with active rheumatoid arthritis was diluted serially and added to 1 per cent suspension of *normal* sheep erythrocytes in one set of tubes, and in another set of tubes the same serum in the same dilutions was added to a similar amount of 1 per cent suspension of *sensitized* sheep erythrocytes. Rose and co-workers[1] report agglutination up to 1+ occurs with the sensitized sheep erythrocytes in a serum dilution that is sixteen times (or more) the

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dilution with the normal sheep erythrocytes. Sheep erythrocytes were sensitized by 2 units of rabbit antisheep cell serum prepared by the technique used for the Kolmer complement fixation test.[2] The normal sheep erythrocytes were prepared from fresh defibrinated blood by washing the cells three times in saline (0.85 per cent), centrifuging, and resuspending in saline to make a 1 per cent suspension by volume.

Dilutions of rabbit antisheep cell sera ranging from 1:1,000 to 1:10,000 were made in saline; 0.5 ml. of each dilution was then mixed with 0.5 ml. of a 2 per cent suspension of washed sheep cells and to each tube 0.1 ml. of a 1:10 dilution of fresh guinea pig complement was added. The tubes were incubated in a water bath at 37° C. for one hour and the highest dilution of the serum producing complete hemolysis was considered to contain 1 unit of hemolysin.

When doing the agglutination test, the rabbit antisheep cell serum was diluted so as to contain 2 units of hemolysin and mixed with an equal volume of a 2 per cent suspension of sheep cells.

This method determines the unit of sensitizing antibody according to its hemolytic titer, but, as will be discussed later, we were unable to obtain differential titers exceeding +8 by this technique. Rose[3] later suggested the agglutinative titer of the serum rather than the hemolytic titer as a more reliable index. Therefore the greater number of our determinations were made with the agglutinative titer.

For the agglutinative titer, dilutions of rabbit antisheep cell serum from 1:100 up, in steps of 100, in a volume of 0.25 ml. were set up; to each tube 0.25 ml. of a 2 per cent suspension of sheep erythrocytes was added; the tubes were incubated at 37° C. for one hour and then kept in the icebox overnight. After centrifugation in order to obtain more definite agglutination, readings were taken. The highest serum dilution giving visible agglutination was taken as the agglutinative titer. For example, if the titer were 1:500, a dilution of 1:1,000 was used.

Whether the hemolytic or agglutinative titer was used the remainder of the test was set up identically. Serum was obtained from freshly collected venous blood of patients and the complement was inactivated by heating at 56° C. for thirty minutes. Dilutions of serum in duplicate (twelve tubes each) containing 0.25 ml. saline were made. To each of one series was added 0.25 ml. of 1 per cent suspension of normal sheep erythrocytes and to the other series 0.25 ml. of the 1 per cent suspension of sensitized sheep erythrocytes. The final serum dilutions were therefore 1:4, 1:8, ... 1:8,192. These were mixed well and placed in the icebox and read in twenty-four hours. In reading the results the highest dilution of serum showing a 1+ agglutination was recorded as the end point. The differential titer was the highest dilution of the serum agglutinating sensitized sheep erythrocytes to 1+ divided by the highest dilution of the serum agglutinating normal sheep cells to 1+. For example, if sensitized sheep cells were agglutinated to 1+ in a serum dilution of 1:128 and the normal sheep cells agglutinated to 1:16, then the differential titer would be 8. (This can be referred to as the "algebraic difference.")

In obtaining material for this study it was decided to include all cases of arthralgia and cases of the so-called collagen diseases. But after examining twelve patients it was apparent that the differential titers being obtained were not high enough to be significant. At this time the technique was reviewed and the decision made to use the agglutinative titer. Thereafter, cases of rheumatoid arthritis, osteoarthritis, rheumatoid spondylitis, psoriatic arthritis, rheumatic fever, lupus erythematosus disseminatus, and controls were used, but the primary interest was in active rheumatoid arthritis so that the reproducibility of the test could be determined.

RESULTS

Using the hemolytic titer, agglutination tests were performed with the sera of twelve patients. The results are tabulated in Table I. The differential titer never exceeded an eightfold difference even though this includes four cases of active rheumatoid arthritis. There was no correlation between activity and the differential titer.

TABLE I. DIFFERENTIAL SHEEP CELL AGGLUTINATION USING THE HEMOLYTIC TITER

PATIENT	DIAGNOSIS	SEX	AGE (YR.)	SED. RATE	W.B.C.	N.S.C.	S.S.C.	DIFF. TITER
E. K.	Rheumatoid arthritis active, with scleroderma and calcinosis	F	64	23	8,800	1:4	1:8	2
J. H.	Rheumatoid arthritis active; osteoarthritis	F	65	25	8,800	1:4	1:4	0
R. H.	Rheumatoid arthritis active	F	54	23	8,500	1:16	1:16	0
J. S.	Rheumatoid arthritis active	M	54	24	9,100	1:16	1:128	8
M. M.	Rheumatoid arthritis inactive	M	75	9	7,400	0	0	0
M. S.	Lupus erythematosus disseminatus	F	20	24	800	1:16	1:32	2
E. S.	Palindromic rheumatism	F	40	13	9,000	1:4	1:8	2
R. M.	Acute rheumatic fever; pregnancy (2 mo.)	F	24	26	14,400	0	1:4	4
L. K.	Undiagnosed joint pain	M	29	25	14,800	1:4	1:16	4
B. B.	Bilateral sclerosing osteitis of sacrum	M	19	5	7,700	0	1:4	4
J. F.	Infectious mononucleosis	F	23	-	7,500	1:128	1:512	4
G. L.	Conversion hysteria with joint pain	F	24	7	5,800	1:8	1:16	2

N.S.C., Normal sheep cells.
S.S.C. Sensitized sheep cells.

Using the agglutinative titer, agglutination tests were performed on the sera of forty-four patients and the results are summarized in Table II. The cases may be divided into the following groups: (1) active rheumatoid arthritis, (2) nonrheumatoid joint disease, (3) latent syphilis, and (4) controls.

The differential titers in the twenty-one cases of active rheumatoid arthritis ranged from 0.25, in which the normal sheep cells were agglutinated to 1:16 and the sensitized sheep cells to 1:4, to the highest differential of 64. These patients all had elevated sedimentation rates, joint pain, and objective evidence of joint disease. The magnitude of the difference in titer did not reflect the clinical activity of the disease nor was there any definite correlation between the differential titer and the erythrocyte sedimentation rate. It is interesting to note that one of the controls had a differential titer of 64. An average of the differential titers of the twenty-one cases of active rheumatoid arthritis is 21.56 which is above the sixteen fold difference which has been designated by Rose and co-workers[1] as the upper limit of "normal." Table III summarizes these averages.

TABLE II. DIFFERENTIAL AGGLUTINATION WITH AGGLUTINATIVE TITER—FORTY-FOUR PATIENTS

CLINICAL DIAGNOSIS	NUMBER OF CASES	DIFFERENTIAL AGGLUTINATION TITERS							
		0	2	4	8	16	32	64	128
Rheumatoid arthritis active	21	3		3	1	7	4	3	
Rheumatoid spondylitis	2				2				
Rheumatoid spondylitis with peripheral joint involvement	1				1				
Psoriatic arthritis	2	1				1			
Rheumatic fever—acute	3		1	2					
Lupus erythematosus disseminatus	1			1					
Osteoarthritis	1							1	
Infectious arthritis	1		1						
Syphilis	5				1	3	1		
Controls	7	2		3	1			1	

TABLE III. AVERAGES OF DIFFERENTIAL TITERS—FORTY-FOUR PATIENTS

CLINICAL DIAGNOSIS	NUMBER OF CASES	DIFFERENTIAL TITER AVERAGES						
		0	2	4	8	16	32	64
Rheumatoid arthritis active	21				21.56			
Rheumatoid spondylitis	2				8			
Rheumatoid spondylitis with peripheral joint involvement	1				8			
Psoriatic arthritis	2				8			
Rheumatic fever	3			(3.33)				
Lupus erythematosus disseminatus	1			4				
Osteoarthritis	1							64
Infectious arthritis	1		2					
Syphilis	5				17.6			
Controls	7				12			

The cases of nonrheumatoid joint disease included: psoriatic arthritis, two patients; rheumatoid spondylitis, three patients, one of whom had peripheral joint involvement; osteoarthritis, one patient; active rheumatic fever, three patients; infectious arthritis, subsiding after evacuation of an ischiorectal abscess, one patient; and one patient with lupus erythematosus disseminatus. The patient with osteoarthritis had a differential titer of 64. One of the patients with psoriatic arthritis had a differential titer of 16. None of the others had differential titers exceeding 8. The average of the differential titers of this group was 10.9.

Another point of interest is the consistently high differential titer which was obtained with the sera of patients with latent syphilis but without subjective or objective evidence of joint disease. Five cases averaged a differential titer of 17.6 (see Table III) with differentials ranging from eightfold to thirty-two fold.

The seven control patients with no evidence of joint disease and with negative serologies included two cases of psychoneurosis; two normal individuals; one case of pericarditis, etiology undetermined; single cases of heart failure and hypertension. These controls had differential titers ranging from zero to 64 and the average of the differential titers was 12. This indicates that the dilution of the rabbit antisheep cell serum used in our studies was of the proper quality and that its agglutinative titer was satisfactory. Certainly, if reproducible results are to be obtained, such an adequate agglutinative titer is necessary.

It is apparent from the results obtained in this study that sensitized sheep cell agglutination is not specific enough to be of value as a diagnostic aid for active rheumatoid arthritis. Of the twenty-one cases of active rheumatoid arthritis, 66 per cent showed a differential titer of 16 or less. If the upper limit of "normal" were reduced to a differential of 8, 33 per cent of these cases were still "negative." It would not be feasible to lower this dividing line to 8 since

the average differential titer of the control group was 12. As is shown in Table III the average for the active rheumatoid arthritis group was 21.56, which was higher than in any other control or disease group. There is a tendency for the sera of this group of patients to agglutinate sensitized sheep cells to a higher titer, but not sufficiently so to warrant the claim that this test might be specifically applied in any individual case.

Only one control serum had a differential titer above 8, but that was 64. This result might suggest an error in technique or in the quality of the rabbit antisheep cell serum used, but set up at the same time as this control serum and with rabbit antisheep cell serum of the same agglutinative titer were sera from another control, three patients with active rheumatoid arthritis, two with rheumatoid spondylitis, one patient with psoriatic arthritis, and one with syphilis. The differential titers of the rheumatoid arthritis cases were 32, 8, and zero; those of the psoriatic arthritis and syphilis cases were each 16; those of the rheumatoid spondylitis cases were each 8; and that of the other control was 4.

DISCUSSION

Since the results obtained in this study indicate that the sensitized sheep cell test is not specific enough to be of value in the diagnosis of active rheumatoid arthritis, it is difficult to postulate what the mechanism might be.

Rose and associates[1] carried out differential sheep cell agglutination tests with the electrophoretically separated beta-gamma globulin fractions of the serum from patients with active and inactive rheumatoid arthritis and found that the results paralleled those which they obtained with the whole serum. They also found that, in general, the patients with active rheumatoid arthritis with differential titers of 16 or higher also gave positive streptococcal agglutination tests.

Wallis[4] has suggested that the agglutination of streptococci by the serum of patients is a nonspecific effect, since the same serum may also agglutinate collodion particles. He also noted the increased ability of the serum of patients with rheumatoid arthritis to agglutinate nonencapsulated pneumococci[5] and suggested that this might be due to a nonspecific enhancement of the action of normally present antibodies. It also has been shown[4] that the sera of patients with well-established rheumatoid arthritis have a tendency to precipitate in the saline control tube of a precipitin test, especially after centrifugation.

In this study no attempts were made to perform differential sheep cell agglutinations on serum fractions since consistent results were not obtained with the whole serum.

The mechanism of this reaction is very puzzling. It appears likely that the reaction is nonspecific in the sense that the reaction is not due to the presence of a specific antibody in the patient's serum but to an increase in some component, possibly globulin, which alters the physicochemical equilibrium.

SUMMARY AND CONCLUSIONS

1. Using the hemolytic titer as proposed by Rose and co-workers,[1] agglutination tests with sensitized and normal sheep cells were performed with the sera

of twelve patients. The differential titer never exceeded an eightfold difference even though four cases of active rheumatoid arthritis were included.

2. Using the agglutinative titer, agglutination tests were performed with the sera of forty-four patients including twenty-one patients with active rheumatoid arthritis. The magnitude of the difference in titer did not reflect the clinical activity of rheumatoid arthritis nor were individual determinations significantly different in the sera of patients with rheumatoid arthritis as compared with various control groups.

3. Differential agglutination of normal and sensitized sheep erythrocytes, using either the hemolytic or agglutinative titer, is not specific enough to be of value as an aid in the diagnosis of active rheumatoid arthritis.

REFERENCES

1. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O.: Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of Patients With Rheumatoid Arthritis, Proc. Soc. Exper. Biol. & Med. **68**: 1, 1948.
2. Kolmer, J. A.: Clinical Diagnosis by Laboratory Examinations, New York, 1944, D. Appleton-Century Company, Inc.
3. Rose, H. M.: Personal communication.
4. Wallis, A. D.: Rheumatoid Arthritis, Non-Specific Serologic Reactions, Am. J. M. Sc. **212**: 716, 1946.
5. Wallis, A. D.: Rheumatoid Arthritis, The Pneumococcus Antibodies, Am. J. M. Sc. **212**: 718, 1946.

EVALUATION OF AN IN VITRO HEPARIN TOLERANCE TEST FOR THROMBOEMBOLIC DISEASE

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THE need for a laboratory test to predict thromboembolic disease has been recognized for some time. Waugh and Ruddick,[1, 2] working along these lines, measured the ability of whole blood to counteract the anticoagulant effects of heparin in vitro. The basis for this test was the physiologic antagonism between thromboplastin and heparin; the former substance was considered to be increased in the bloodstream in cases of accelerated coagulation. By using increasing amounts of heparin in a series of test tubes, they attempted a "controlled declaration of the clotting process." In interpreting the Waugh-Ruddick test, any curve that falls below the accepted normal range would indicate accelerated coagulability. On the other hand, results above the normal range would point to delayed coagulability.

Silverman[3] modified the test in an attempt at increasing its accuracy and eliminating certain inherent mechanical difficulties. In our studies at The Bronx Hospital, the Silverman modification of the Waugh-Ruddick test was used.

METHOD

Serial dilutions of heparin were prepared as follows.

A stock solution was made containing 0.6 c.c. of heparin (1,000 units per cubic centimeter) in 300 c.c. of normal saline. The actual dilutions were made from the stock solution.

	STOCK SOLUTIONS (C.C.)	NORMAL SALINE (C.C.)	HEPARIN (UNIT PER 0.1 C.C.)
1.	0	50	0
2.	5	45	1/50
3.	10	40	2/50
4.	15	35	3/50
5.	20	30	4/50
6.	25	25	5/50
7.	30	20	6/50
8.	35	15	7/50

Four and one half cubic centimeters of blood were drawn and added to a tube containing 0.5 c.c. of 0.1M sodium oxalate. This was centrifuged to separate the plasma; 0.1 c.c. of plasma was placed in each of eight tubes (100 by 13 mm.). To each tube 0.1 c.c. of one of the correspondingly numbered heparin solutions was added. Then 0.2 c.c. of 0.01M calcium chloride was added to each tube to offset the oxalate previously used and the time was noted. All tubes were then immediately placed in a water bath at 37.5° C. and the rack was gently tilted periodically until coagulation was noted. Invariably the numbered tubes clotted in the expectedly orderly sequence.

The following precautions were taken to insure accuracy and uniformity of the test.

1. Stock solutions were kept at a temperature of 4° C. when not in use.
2. The Wassermann type of tubes were of uniform size and scrupulously clean.
3. Venepuncture was done carefully and a clean dry syringe and needle were used.

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4. The test was always performed in a water bath of constant suitable temperature as suggested by Whittaker.[4]

5. At periodic intervals the heparin solutions were checked for stability. Determinations were performed on the plasma of a normal individual using both old and freshly prepared solutions. At intervals of two to three weeks these results checked closely. Likewise, a new unopened vial of heparin (1,000 units per cubic centimeter) was checked against the old one and again a close correlation in results was noted.

6. The test was performed at about 10 A.M. in each instance.

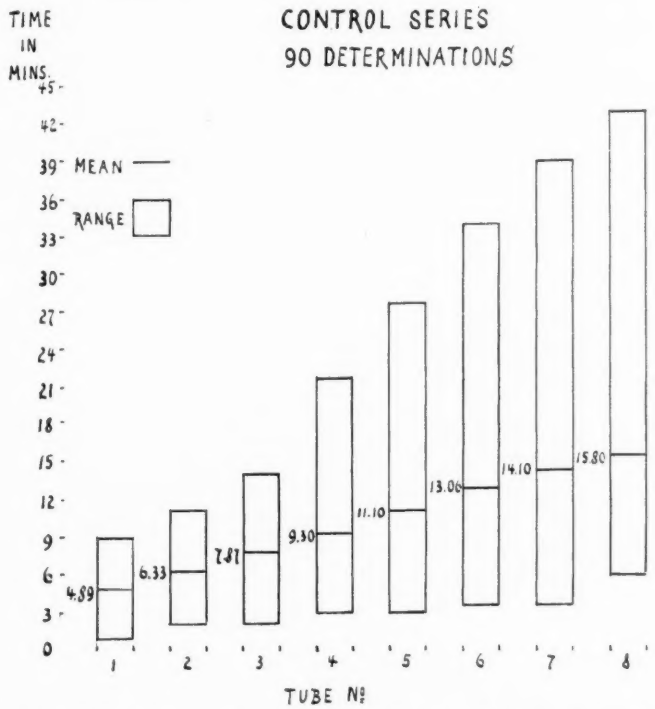

Fig. 1.—This graph shows the range and mean clotting times for each tube in ninety control determinations.

RESULTS

Seventy-four individuals were used as controls and ninety determinations were performed on this group. The controls varied in age from the third to the seventh decade and none showed clinical evidence of heart disease, thromboembolic phenomena, or blood dyscrasias. All were ambulatory. Our findings with this group are recorded in Fig. 1. It will be noted that as the tubes contain increasing amounts of heparin the mean clotting time increases. The average increase in clotting time from tube to tube is 1.56 minutes, with an actual range of difference from 1.04 minutes to 1.80 minutes. Attention is directed to the wide scatter of clotting times among normal individuals, especially in the tubes containing larger amounts of heparin. Two and three determinations were performed on some of the controls to note the variation in heparin tolerance not only among different individuals but also in the same person from day to day.

A few individuals showed a close correlation on repeated determinations, while most showed marked variations. Fig. 2 illustrates the different types of results obtained in two controls.

After having established the normal range, the test was performed on twelve obstetric patients and seven candidates for prostatectomy. In the obstetric cases a determination was made ante partum and forty-eight hours post partum,

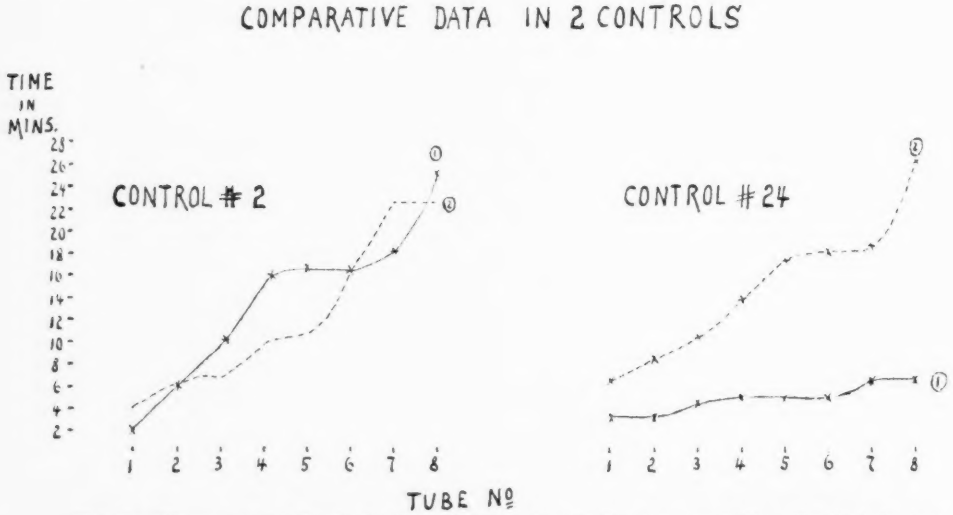

Fig. 2.—These curves illustrate the variation in results upon repeating the test in two controls on different days. In Control 2 the correlation is good; while in Control 24 a marked difference in coagulability is noted. The solid line, *1*, in each control represents the first study; the interrupted line, *2*, is the repeat study.

and if an accelerated curve was then found a third test was performed seven days post partum. The same routine was followed in the prostatectomy cases. The initial determination in each case was included in our control series. Table I shows comparison of these groups and our controls, with a statistical analysis of the results. The difference between the standard deviations of the control group and the obstetric postoperative group is smaller than 3 sigma, in the case of each of the eight tubes. Thus, none of the tubes shows a significant statistical difference from the control to the obstetric-postoperative group.

TABLE I. STATISTICAL ANALYSIS OF MEAN DIFFERENCES OF SERIAL CLOTTING TIMES BETWEEN CONTROL AND POSTOPERATIVE GROUPS

	CONTROLS (90)		POSTOPERATIVE PATIENTS (19)		ANALYSIS	
TUBE	MEAN CLOTTING TIME*	STANDARD DEVIATION	MEAN CLOTTING TIME	STANDARD DEVIATION	DIFFERENCES BETWEEN MEANS	TEST FOR SIGNIFICANCE (3σ)
1	4.87	1.77	4.04	1.56	0.83	1.23
2	6.33	2.01	5.44	1.80	0.89	1.35
3	7.87	2.74	6.59	2.46	1.28	1.86
4	9.30	3.72	7.74	2.79	1.56	2.19
5	11.10	5.10	8.85	4.05	2.25	3.21
6	13.06	6.21	9.85	4.50	3.21	3.63
7	14.10	7.20	10.63	5.40	3.47	4.26
8	15.80	8.76	11.44	6.18	4.36	5.64

*All clotting times expressed in minutes.

DISCUSSION

In clinical application, preliminary studies by Waugh and Ruddick[2] showed that an accelerated type of curve was obtained under the following circumstances.

1. During uncomplicated bed rest.
2. Following operative procedures.
3. In the presence of acute infections.
4. In the presence of hemorrhage.

Prolongation of the curve was noted in patients undergoing radiation therapy.

In performing his test on nine operative patients, Silverman[3] noted a definite increase in coagulability following operation. This began within twenty-four hours after operation and lasted for approximately one week. Concomitant platelet counts were done and no increase was found until six or seven days after operation, so that thrombocytosis was not a significant factor in these results in the early postoperative period.

Further application of the principles of the Waugh-Ruddick test by Ogura and co-workers[5] in twenty-seven cases of coronary thrombosis revealed an accelerated coagulation curve in 77.8 per cent of the cases. The onset and duration of this acceleration corresponded with the time range pointed out by Silverman, as noted. A total of sixteen controls was used in this series; seven were normal individuals and nine were cardiac patients without thrombosis and at bed rest. Conventional tests for coagulation performed alongside this in vitro heparin tolerance test failed to detect any acceleration. The conclusion reached by these authors was that the accelerated coagulation did not cause the coronary thrombosis but was a result of myocardial destruction.

De Takats[6] made use of an in vivo heparin tolerance test as an aid in the diagnosis of thromboembolic disease. Ten milligrams of purified heparin were injected intravenously and coagulation times determined by the capillary tube method before the test and at ten-minute intervals for forty minutes thereafter. He found a group of patients who were resistant to heparin. These were patients in the early postoperative period, patients who had suffered cardiovascular accidents, and patients with Buerger's disease.

The heparin tolerance curves in the post-partum and postoperative states in our patients did not vary significantly from those obtained in the control series. While there may be a difference in the degree of coagulability of the blood of post-partum and postoperative patients as compared with normal individuals, our results fail to demonstrate this. This may possibly be due to inherent inadequacy of the test.

SUMMARY

An in vitro heparin tolerance test for the prediction of thromboembolic disease was studied in seventy-four controls, twelve obstetric patients, and seven patients who had prostatectomies. The results were subjected to statistical analysis. In our hands this test failed to show a significant difference between the control and the post-partum-postoperative groups. This differs from the

reports of previous investigators.[2, 3] In view of this discrepancy, further investigative work with this test and on other factors affecting blood coagulation seems indicated in order to clarify their place in the prediction of thromboembolic disease.

REFERENCES

1. Waugh, T. R., and Ruddick, D. W.: A Test for Increased Coagulability of Blood, Canad. M. A. J. **50**: 547, 1944.
2. Waugh, T. R., and Ruddick, D. W.: Studies On Increased Coagulability of Blood, Canad. M. A. J. **51**: 11, 1944.
3. Silverman, S. B.: A Modification of the Waugh-Ruddick Test for Increased Coagulability of the Blood, and Its Application to the Study of Postoperative Cases, Blood **3**: 147-154, 1948.
4. Whittaker, J.: The Effect of Temperature on the Waugh and Ruddick Test for Increased Coagulability of Blood, Canad. M. A. J. **52**: 185-186, 1945.
5. Ogura, J. H., Fetter, N. R., Blankenhorn, M. A., and Glueck, H. I.: Changes in Blood Coagulation Following Coronary Thrombosis Measured by the Heparin Retarded Clotting Test (Waugh and Ruddick Test), J. Clin. Investigation **25**: 586, 1946.
6. de Takats, G.: Heparin Tolerance, Surg., Gynec. & Obst. **77**: 31, 1943.

OBSERVATIONS ON THE COAGULATION DEFECT IN THROMBOCYTOPENIC PURPURA

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THE presence of an excess of heparin or of a heparin-like substance in the blood of patients with idiopathic or secondary thrombocytopenic purpura and other hemorrhagic states has been postulated by Allen and co-workers.[1] This opinion was developed in part as the result of protamine-heparin titrations and also by clinical observations on the effect of protamine and toluidine blue on abnormal bleeding. These agents are capable of reacting with several endogenous compounds in the body, some of which are heparin-like while others appear to be unrelated to heparin.[2] They were able to show that higher concentrations of protamine were necessary to cause coagulation of heparinized blood from patients with thrombocytopenic purpura than from normal persons. They also noted temporary cessation of hemorrhagic phenomena in some patients following intravenous administration of protamine or toluidine blue in a dosage of 1 to 4 mg. per kilogram of body weight. Attention had been directed to this "heparinoid state" by the hemorrhagic tendency which appeared in dogs following total body irradiation (roentgen ray).[3, 4] Allen and co-workers found in the blood of these hemorrhagic animals a circulating anticoagulant which reacted in many ways similarly to heparin, and the bleeding tendency could be controlled temporarily by the intravenous injection of protamine sulfate or toluidine blue.

Holmgren and Wilander[5] in 1937 found that "acidic" heparin was removed completely from blood in vitro by adding the "basic" dye toluidine blue. This was apparently a precipitation phenomenon. Blood to which heparin is added has a prolonged coagulation time, but the addition of a solution of toluidine blue will cause heparinized blood to clot in a few minutes. Chargoff and Olson[6] observed that the "basic" amine, protamine, had an effect similar to that of toluidine blue but this action was apparently mediated through its effect on the electrical charge of heparin. Jorpes[7] injected a protamine intravenously in man without untoward reaction and from in vitro studies concluded that protamine would neutralize heparin in the proportion of six parts of protamine to ten parts of heparin. Parkin and Kvale[8] concluded, from in vitro studies, however, that protamine neutralized heparin in a one to one ratio. There are several factors involved in blood coagulation through which heparin may act, but the exact mode of its action is not known. Heparin, when added in increasing amounts to normal blood, first shortens and then lengthens the whole blood

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clotting time.[9, 10, 11] The amount of heparin necessary to shorten the clotting time has been shown to vary from 0.0001 to 0.01 unit per cubic centimeter of blood. One-tenth unit of heparin per cubic centimeter of blood will prolong the clotting time. This prolongation may be due to an effect on thrombin, prothrombin, thrombokinase, or possibly on other systems.[12, 13, 14, 15]

The mechanism by which protamine alters the coagulation process has not been determined and it too may act in one of several different ways.[15-19] An antithromboplastic or an antiprothrombic action in the first phase of blood coagulation has been postulated.[14, 15, 16] It also has been suggested that it may have a fibrinoplastic activity in the second phase. In sufficient concentration protamines also have been shown to precipitate fibrinogen and thereby may prolong blood coagulation when given in excess. Utilizing protamine and heparin in an attempt to determine changes in the coagulability of blood, as is done in the protamine titration test, introduces many unknown factors.

METHODS

While utilizing small amounts of heparin for prolonging the coagulation of blood by the method of Waugh and Ruddick,[20, 21] it was noted that some patients who were actively bleeding had an augmented reaction to heparin. The Waugh-Ruddick test was originally devised as a modification of the blood coagulation test of Lee and White[22] to provide a controlled deceleration of the coagulation process. The technique is as follows.[20, 21]

> Nine Wassermann tubes (100 by 13 mm.), thoroughly cleaned and dried, are placed in a rack. Tube number one remains empty until the blood is added. Tube number two contains ½ c.c. of 9/10 per cent normal saline. Serial dilutions of heparin in ½ c.c. of saline are added to the remaining seven tubes so that tube three receives 0.1 unit of heparin in ½ c.c. saline, tube four receives 0.2 unit of heparin in saline, and so on until tube nine receives 0.7 unit of heparin. To each of the tubes is added 1 c.c. of blood drawn with a minimum of trauma, using a number eighteen needle and measured from the same 10 c.c. syringe in which it was drawn. The tubes are stoppered and tipped to approximately 85 degrees at two-minute intervals until coagulation occurs. The time which elapses from the moment blood is first seen in the syringe until coagulation occurs is the end point in that particular tube.

The test has been used to study the increased speed of coagulation of the blood of patients with myocardial infarction and other thrombotic conditions.[23] It has not been used heretofore, to our knowledge, in a study of patients with hemorrhagic tendencies.

RESULTS

The test was performed on thirty normal subjects and the results, as presented in Fig. 1, are in agreement with those of Waugh and Ruddick. The time of coagulation in tubes 1 and 2, which contain no heparin, varied from 4 to 12 minutes. In tube 3, containing 0.1 unit of heparin, coagulation occurred in from 12 to 40 minutes. A gradual increase in the time required for coagulation was found as increasing amounts of heparin are encountered. Coagulation ultimately occurred in from 40 to 90 minutes. In no instance was the blood rendered incoagulable under these conditions. The two extremes in the time of

coagulation in normal subjects are shown by the solid lines in Fig. 1, while individual readings on each of the thirty patients are represented by the dots.

When the Waugh-Ruddick test was tried on the blood of patients who were actively bleeding because of thrombocytopenic purpura, it was noted that there was an increased sensitivity to heparin as indicated by an increased time for coagulation in the tubes containing this material. Consequently the test was performed on fifteen patients who had bleeding tendencies associated with pri-

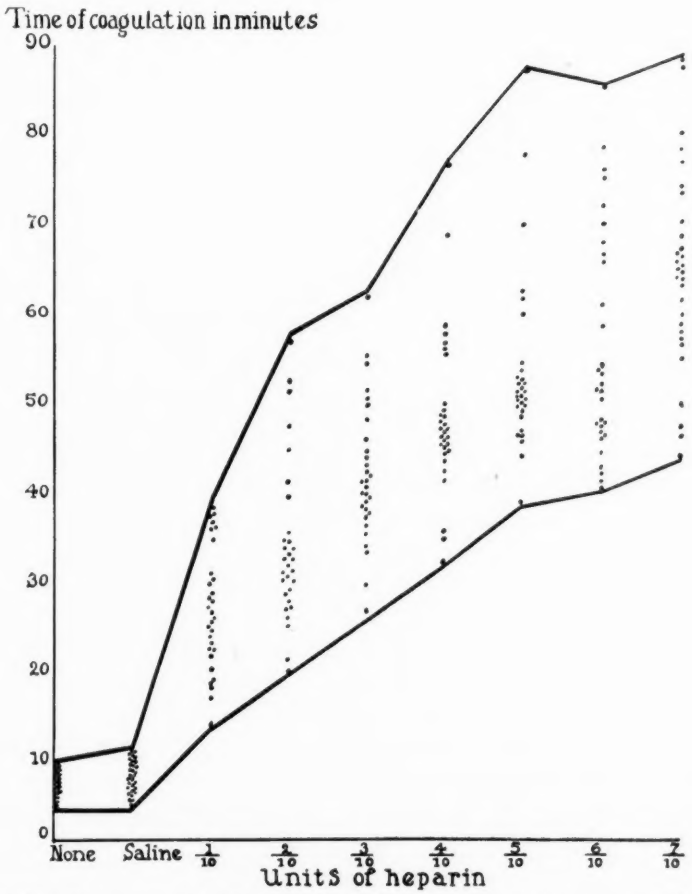


Fig. 1.—Showing the response to the Waugh-Ruddick test in thirty normal individuals. The lines represent the two extremes.

mary or secondary thrombocytopenia. The patients and their diagnoses are listed in Table I, nine of them being classified as idiopathic thrombocytopenic purpura. The laboratory findings were typical in all cases: the clotting time was essentially normal; there was prolongation of the bleeding time; the thrombocytes were diminished, clot retractility was absent; and they presented clinical manifestations of hemorrhage. In three patients the thrombocytopenia was secondary to leucemia. It was secondary to a chemical agent in Case 13, and in Case 14 autopsy revealed a diffuse reticuloendotheliosis. Patient J. B., Case

TABLE I

CASE	PATIENT	CAUSE	PLATELETS (THOUSANDS)	BLEEDING TIME (MIN.)	CLOTTING TIME (MIN.)	CLOT RETRACTILITY	HEMORRHAGIC PHENOMENA
1.	D. M.	Idiopathic	8.0	10+	5.0	None	Yes
2.	W. M.	Idiopathic	5.0	7.	5.0	None	Yes
3.	M. S.	Idiopathic	19.0	15+	4.0	None	Yes
4.	J. G.	Idiopathic	4.0	10+	6.0	None	Yes
5.	M. P.	Idiopathic	2.0	10+	5.0	None	Yes
6.	J. S.	Idiopathic	5.0	10+	5.0	None	Yes
7.	R. P.	Idiopathic	10.0	10+	5.5	None	Yes
8.	S. L. L.	Idiopathic	8.0	10+	6.0	None	Yes
9.	L. B.	Idiopathic	12.0	10+	6.25	None	Yes
10.	J. R.	Lymphatic leucemia	5.0	10+	8.0	None	Yes
11.	F. S.	Myelogenous leucemia	36.0	20+	6.0	None	Yes
12.	W. J. W.	Monocytic leucemia	2.0	10+	6.0	None	Yes
13.	F. R.	? Toxic agent	4.0	10+	3.5	None	Yes
14.	C. D.	Reticuloendothelial hyperplasia	5.0	10+	4.5	None	Yes
15.	J. B.	Aplastic anemia	2.0	8.5	4.0	None	Yes

15, had aplastic anemia. One of the nine patients with idiopathic thrombocytopenic purpura died of cerebral hemorrhage before splenectomy could be performed. Splenectomy was done in eight patients, seven of whom responded satisfactorily with a return of the platelet count and bleeding time to normal and cessation of the hemorrhagic tendency. The eighth patient responded temporarily but reverted to the previous hemorrhagic state.

The studies on these patients with thrombocytopenic purpura revealed an essentially normal coagulation time in the first two tubes, which do not contain heparin, but a markedly prolonged coagulation time in the remaining tubes containing serial dilutions of heparin. Fluctuations were noted in the speed of coagulation in individual patients from time to time when there was no significant change in the number of platelets, but when there were active clinical hemorrhagic tendencies the increased sensitivity to heparin was always present. The variations in the Waugh-Ruddick test more nearly paralleled the clinical manifestations of hemorrhage than did the platelet level.

Fifty-six determinations were done on these fifteen patients and the results are presented in Fig. 2. The solid line is a typical Waugh-Ruddick curve in a normal subject, presented for comparison. Coagulation occurred in 20 minutes in the presence of 0.1 unit of heparin, in 32 minutes with 0.2 unit, in 46 minutes with 0.3 unit, and in 84 minutes with 0.7 unit of heparin in the last tube. The shaded area in the figure represents the extremes in the response in fifty-six determinations on fifteen patients while they were actively bleeding. In these patients coagulation required from 45 to 100 minutes in the presence of 0.1 unit of heparin although the coagulation time in the first two tubes, containing no heparin, had been essentially normal. In the fourth tube, containing 0.2 unit of heparin, coagulation occurred in 60 minutes in one patient, but in many instances the blood was incoagulable in this dilution of heparin and in all subsequent dilutions. The shortest coagulation time with 0.3 unit of heparin was 80

minutes, but in most instances the blood was incoagulable with this dilution of heparin. There was a progressive lengthening of the coagulation time as the concentration of heparin was increased, and even in those having the shortest coagulation time it was much longer than the normal and an incoagulable state was reached in all cases. In none of the normal subjects was the blood made incoagulable by these dilutions of heparin. All determinations fell between the extremes represented by the shaded area. Incoagulability of the blood was usually produced with 0.3 to 0.4 unit of heparin and it remained incoagulable

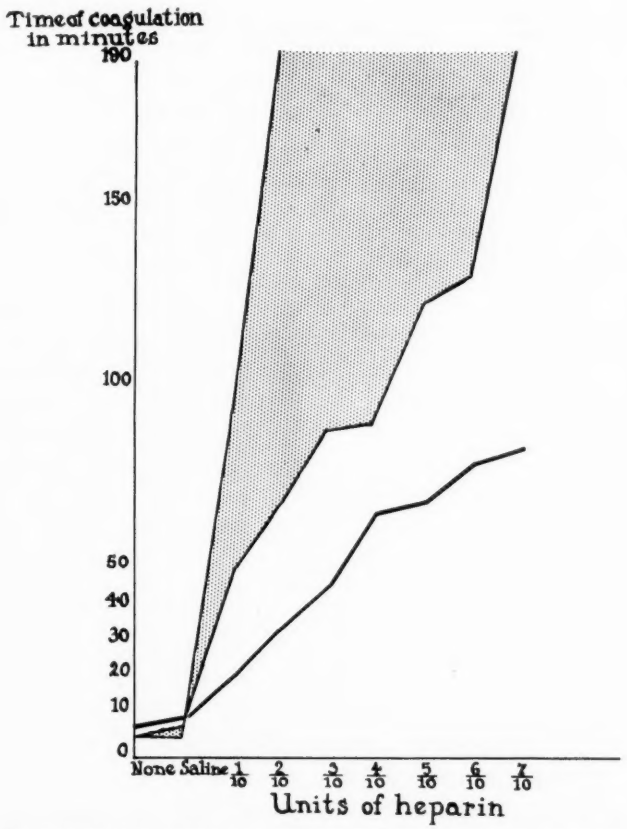


Fig. 2.—The heavy line shows the Waugh-Ruddick curve in a normal person. The shaded area represents the extremes in the response obtained in thrombocytopenic patients while actively bleeding. Blood from these patients is rendered incoagulable by this small amount of heparin. Although the chart is stopped at 190 minutes, all blood samples were observed for twelve or more hours and were still uncoagulated.

in subsequent tubes. These results show that the coagulation time in such patients is normal in the absence of heparin but is markedly increased with even small amounts of heparin and the blood is easily rendered incoagulable.

In four patients with purpura simplex (nonthrombocytopenic purpura) the Waugh-Ruddick test was normal.

Protamine sulfate and toluidine (1 to 4 mg. per kilogram of body weight) were administered to seven patients and their blood was studied in serial dilutions of heparin. Samples of blood were drawn prior to the administration of

protamine or toluidine blue, ten minutes after the administration was completed, and daily thereafter for variable periods. In two patients there was no demonstrable effect. In a third subject the Waugh-Ruddick curve returned to normal following the injection of protamine sulfate, but since the results persisted for more than one week it seems probable that the material was given concomitantly with a spontaneous remission. In the remaining instances the administration of protamine sulfate or toluidine blue apparently shortened the coagulation time in the tubes containing 0.1 and 0.2 unit of heparin and occasionally in the tube

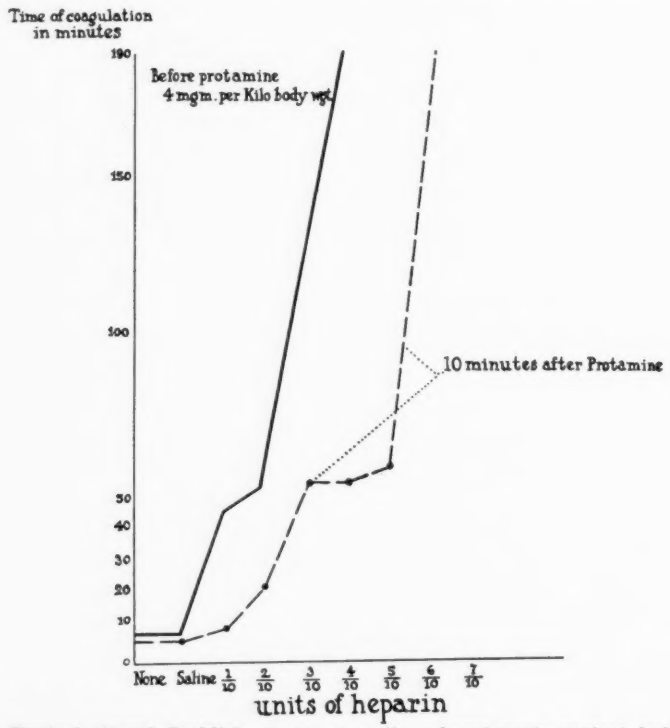


Fig. 3.—Typical Waugh-Ruddick curves on a thrombocytopenic patient before and after the administration of protamine sulfate. The blood did not clot and was observed for twelve hours although the chart is stopped at 190 minutes.

containing 0.3 unit of heparin but had no effect on the tubes containing a higher concentration. In these tubes the blood remained incoagulable. This suggests that the protamine in the blood stream was sufficient to neutralize 0.1 to 0.3 unit of heparin in tubes 3 and 4 but not larger amounts. Fig. 3 shows a typical response in a patient before and after the administration of protamine. The partial flattening of the curve, apparently due to the presence of circulating protamine, was not noted in the twenty-four hour sample.

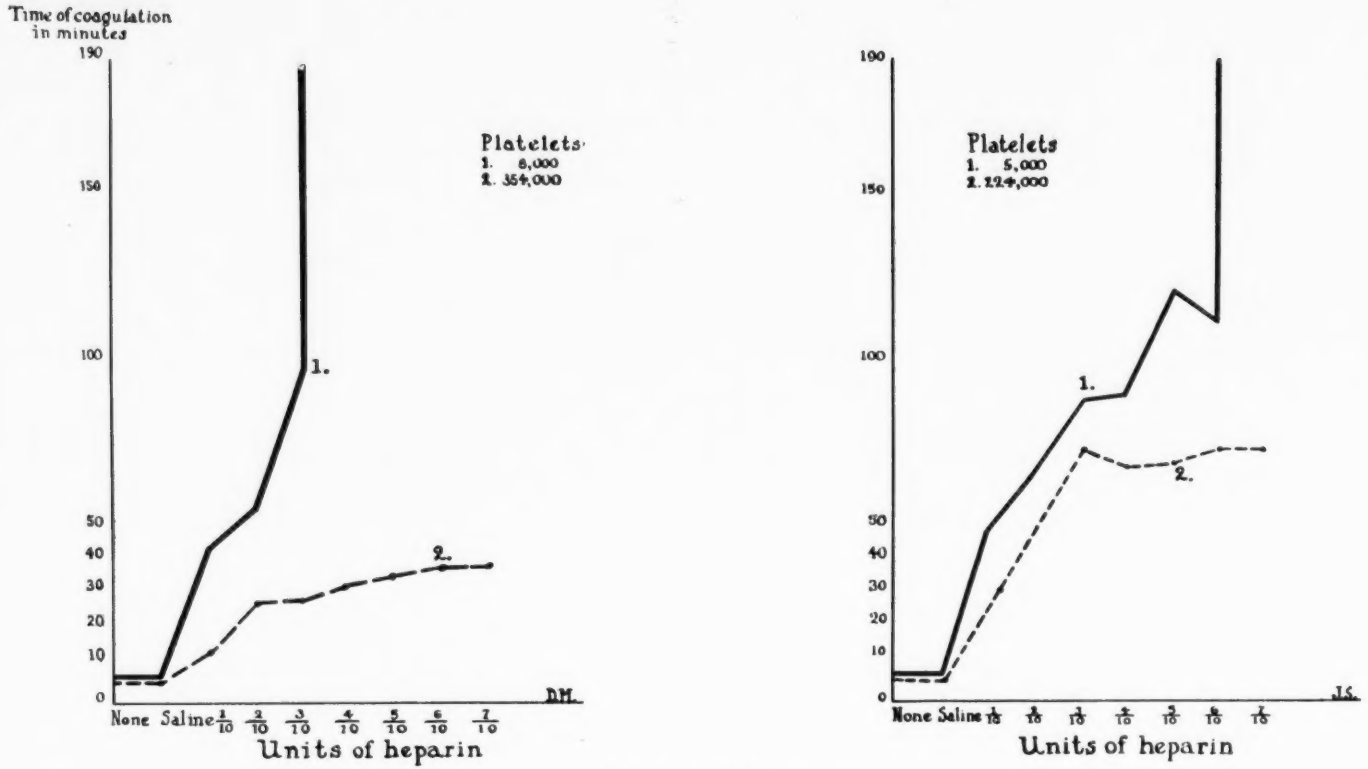
The Waugh-Ruddick test was done on five patients before and after splenectomy. In four of the cases the operation was followed by a complete clinical remission and a return of the platelet count and other laboratory findings to normal levels. In all of these patients the Waugh-Ruddick test also returned to normal. Fig. 4 shows the results of this test in the four cases before and after

splenectomy. In the fifth case the operation was followed by an immediate but moderate rise in the platelet count and a normal Waugh-Ruddick test. Two months later cutaneous hemorrhages had reappeared, the platelet count had dropped to its previous low level, and the Waugh-Ruddick test was again even more prolonged than initially. Fig. 5 shows the Waugh-Ruddick test before operation, one hour postoperatively, and two months later in the patient responding only temporarily to splenectomy.

DISCUSSION

These experiments show that the blood from patients with thrombocytopenic purpura exhibits an unusual sensitivity to heparin in that coagulation is extremely prolonged in its presence and the blood is rendered incoagulable when very small amounts of heparin are added. The prolongation of the coagulation time is less marked with normal blood and it does not reach an incoagulable stage under these conditions. This reaction is not like that produced by the intravenous injection of commercial heparin. The coagulation time of the whole blood as determined by the Lee-White and capillary tube methods is not significantly prolonged in thrombocytopenic patients as is true when an excessive amount of commercial heparin is given. Toluidine blue and protamine sulfate when given to these patients intravenously does not neutralize the incoagulability of the blood in the Waugh-Ruddick procedure as would be expected if excess heparin were the basis for these changes. These substances appear only partially to neutralize the effect of the heparin used in the test. Further evidence against the presence of an excess of heparin in the blood of these patients was suggested by the administration of commercial heparin to normal subjects. The Waugh-Ruddick curves in these individuals are not the same as curves obtained in thrombocytopenic patients.[24] When heparin is given intravenously to a normal person in small amounts (10 mg.), the coagulation time is only slightly prolonged and the blood does not become incoagulable in the Waugh-Ruddick test containing the higher concentrations of heparin. When heparin is given in larger amounts (50 mg.), the coagulation time is definitely prolonged but still the blood does not become incoagulable in the Waugh-Ruddick test. This reaction differs from that of the blood of thrombocytopenic patients in that their blood always becomes incoagulable when tested during a period of hemorrhage and the Waugh-Ruddick curve is entirely different from that of blood heparinized in vivo. These observations agree with the conclusion of Conley, Hartmann, and Morse[25, 26] that the increased susceptibility of thrombocytopenic blood to heparin apparently is not due to an increased amount of circulating heparin-like substance.

The blood of patients with thrombocytopenic purpura is more sensitive to heparin in vitro than the blood of normal persons. This sensitivity closely parallels the clinical manifestations of hemorrhage and follows the clinical state more closely than it does the platelet count. The parallelism between the clinical manifestations of hemorrhage and heparin sensitivity is also closer than that between the clinical manifestations and the platelet count.



1. Before Splenectomy
2. After Splenectomy

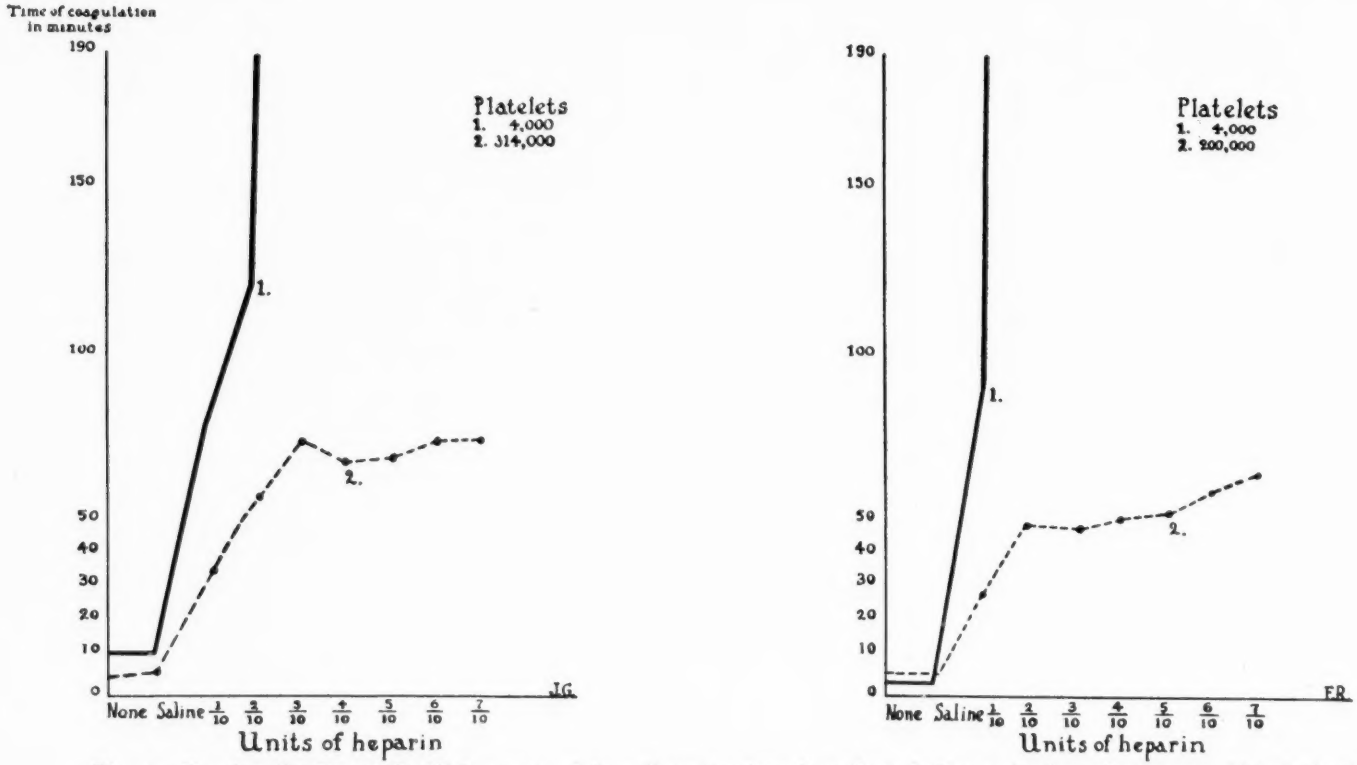


Fig. 4.—Showing the Waugh-Ruddick curves of four thrombocytopenic patients before and after splenectomy. Note lack of clotting prior to operation.

SUMMARY

1. The Waugh-Ruddick test has been used to study the coagulation defect in patients with thrombocytopenic purpura.

2. The blood from such patients is unusually sensitive to small amounts of heparin in vitro.

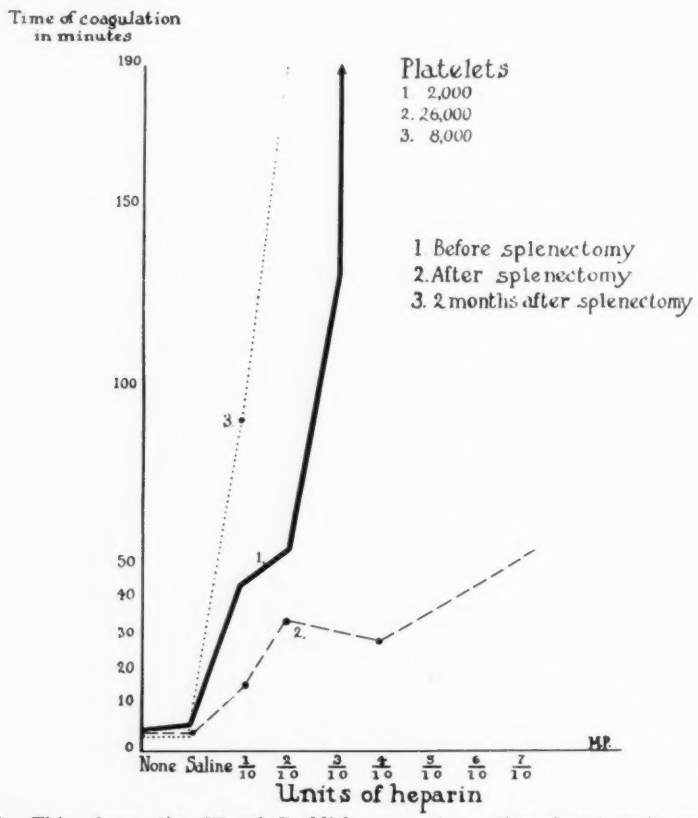


Fig. 5.—This shows the Waugh-Ruddick curve in a thrombocytopenic patient before splenectomy, one hour postoperatively, and two months later at which time bleeding had recurred.

3. The increased sensitivity of the blood to heparin parallels closely the clinical manifestations of hemorrhage.

4. The coagulation defect does not appear to be due to an excess of heparin in the circulating blood.

REFERENCES

1. Allen, J. G., Bogardus, George, Jacobson, L. O., and Spurr, C. L.: Some Observations on Bleeding Tendency in Thrombocytopenic Purpura, Ann. Int. Med. **27:** 382, 1947.
2. Allen, J. G., Grossman, Burton, J., Elghammer, Richard M., Moulder, Peter V., McKeen, Charles L., Jacobson, Leon O., Pierce, Mila, Smith, Taylor R., and Croskie, James M.: Abnormal Bleeding, J. A. M. A. **139:** 1251-1254, 1949.
3. Allen, J. G., and Jacobson, L. O.: Hyperheparinemia: Cause of Hemorrhagic Syndrome Associated With Total Body Exposure to Ionizing Radiation, Science **105:** 388-389, 1947.

4. Allen, J. G., Sanderson, M., Nielham, M., Kirschon, A., and Jacobson, L. O.: Heparinemia (?) Anticoagulant in the Blood of Dogs With Hemorrhagic Tendency After Total Body Exposure to Roentgen Rays, J. Exper. Med. **87**: 71-86, 1948.
5. Holmgren, Hjalmar, and Wilander, Olaf: Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen, Ztschr. f. mikr.-anat. Forsch. **42**: 242-278, 1937.
6. Chargoff, Erwin, and Olson, K. B.: Studies on the Chemistry of Blood Coagulation. VI. Studies on the Action of Heparin and Other Anticoagulants. The Influence of Protamine on the Anticoagulant Effect in Vivo, J. Biol. Chem. **122**: 153, 1939.
7. Jorpes, J. E.: Neutralization of Action of Heparin by Protamine, Lancet **2**: 957, 1939.
8. Parkin, T. W., and Kvale, W. F.: Neutralization of Heparin With Protamine (Salmine), J. Lab. & Clin. Med. **32**: 1396, 1947.
9. Fischer, A., and Astrup, T.: Stöchiometrische Bindungsverhältnisse zwischen Heparin und Gerinnungsstoff, Biochem. Ztschr. **278**: 326-333, 1935.
10. Cronkite, E. P., and Ullrich, F. W.: Project N. M.-007039, Report No. 5, Naval Medical Research Institute, 1948.
11. Begamy, A., and Seiftes, J.: Studies on a Heparin-like Compound, Algarin, Federation Proc. **7**: 206, 1948.
12. Astrup, T., and Darling, S.: Antithrombin and Heparin, Acta physiol. Scandinav. **5**: 13-30, 1943.
13. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: A Quantitative Study on Blood Clotting: Prothrombin Fluctuations Under Experimental Conditions, Am. J. Physiol. **114**: 667, 1936.
14. Brinkhous, K. M., Smith, H. P., Warner, E. D., and Seegers, W. H.: The Inhibition of Blood Clotting: An Unidentified Substance Which Acts in Conjunction With Heparin to Prevent the Conversion of Prothrombin Into Thrombin, Am. J. Physiol. **125**: 683-687, 1939.
15. Ferguson, J. H.: The Action of Heparin, Serum Albumin (Crystalline), and Salmine on Blood-Clotting Mechanisms (in Vitro), Am. J. Physiol. **130**: 759-770, 1940.
16. Tocantins, L. M.: Cephalin, Protamine and Antithromboplastic Activity of Normal and Hemophilic Plasma, Proc. Soc. Exper. Biol. & Med. **54**: 94-97, 1943.
17. Chargoff, E.: The Protamine Salts of Phosphatides, J. Biol. Chem. **125**: 661-670, 1938.
18. Chargoff, E.: Studies on the Chemistry of Blood Coagulation. VII. Protamines and Blood Clotting, J. Biol. Chem. **125**: 671-676, 1938.
19. Nylon, E., Winternitz, M. C., and de Süto-Nagy, J. J.: The Determination of Fibrinogen With Protamine, J. Biol. Chem. **143**: 21-27, 1942.
20. Waugh, Theo R., and Ruddick, D. W.: A Test for Increased Coagulability of the Blood, Canad. M. A. J. **50**: 547-549; **51**: 11-17, 1944.
21. Waugh, Theo R., and Ruddick, D. W.: The Effect of Temperature on the Waugh and Ruddick Test for Increased Coagulability of Blood, Canad. M. A. J. **52**: 185-186, 1945.
22. Lee, Roger I., and White, P. D.: A Clinical Study of the Coagulation Time of Blood, Am. J. M. Sc. **145**: 495-503, 1913.
23. Ogura, J. H., Felter, N. R., Blankenhorn, M. A., and Glueck, H. I.: Changes in Blood Coagulation Following Coronary Thrombosis Measured by the Heparin Retarded Clotting Test (Waugh and Ruddick Test), J. Clin. Investigation **25**: 586-596, 1946.
24. Theilen, E., Fowler, W. M., and Carr, T. L.: Unpublished observations.
25. Conley, C. Lockard, Hartmann, Robert C., and Morse, William I. II: Circulating Anticoagulants: A Technique for Their Detection and Clinical Studies, Bull. Johns Hopkins Hosp. **84**: 255-268, 1949.
26. Conley, C. Lockard, Hartmann, Robert C., and Morse, William I. II: The Clotting Behavior of Human "Platelet-Free" Plasma: Evidence for the Existence of a "Plasma Thromboplastin," J. Clin. Investigation **28**: 340-352, 1949.

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SERUM GLUTAMIC ACID LEVELS AND THE OCCURRENCE OF NAUSEA AND VOMITING AFTER THE INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES

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ONE of the difficulties in the intravenous use of protein digests is the occurrence of anorexia, nausea, and vomiting following rapid infusion. Mixtures of amino acid, on the other hand, have been given at rapid rates with few ill effects. Earlier work with both dogs[1, 2, 3, 4] and man[5] has indicated that the dicarboxylic amino acids, glutamic and aspartic acids, are the major factors in protein hydrolysates responsible for these undesirable reactions. In order to obtain more direct evidence concerning this relationship, the free glutamic acid† content was determined in the serum of subjects receiving the various amino acid preparations before and at the end of the infusion. The free glutamic acid content in the various preparations was also determined. A series of subjects received solutions of partially neutralized glutamic acid in saline, and estimates of the serum free glutamic acid content were made during this infusion. The observations which form the basis for this report indicate a direct correlation between the glutamic acid content of the various preparations and the incidence of nausea and vomiting. In addition, data concerning the quantitative relation of serum free glutamic acid and the occurrence of nausea and vomiting are presented.

METHODS

Three groups of patients were selected for study: Group A (forty-seven subjects) received five different amino acid preparations intravenously (three protein hydrolysates, two amino acid mixtures), all of known glutamic acid content; Group B (thirty-one subjects) received solutions containing glutamic acid which had been partially neutralized; Group C (eleven subjects) received an amino acid mixture fortified with glutamic acid sufficient to bring the content of this dicarboxylic acid up to that present in the acid hydrolysate of casein used in this study. All of the subjects in this study were men chosen at random from the wards of a general medical service. With very few exceptions each subject received only a single infusion.

The amino acid preparations that Group A received are designated by numbers I, II, III, V, and VI.‡ Preparation I was an enzymatic digest of casein; Preparation II was an

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†In this presentation the term "free glutamic acid" when used to express the content of the material in serum refers to the microbiologically available glutamic acid, and probably includes some of the glutamine.

‡The numbers of the amino acid preparations, except for number VI, are the same as used in a previous publication[6] from this laboratory.

acid digest of casein fortified with tryptophane, and Preparation III was a mixture of synthetic amino acids consisting of the ten "essential" amino acids plus glycine. Preparation V, a mixture of amino acids, was formed by the recombination and fortification of fractions of a casein digest. Preparation VI was an enzymatic digest of beef blood proteins.* The volume of the unit dose for Preparations II, III, and V was 500 c.c., for Preparation I, 400 c.c., and for VI, 1,000 cubic centimeters.

In studying the blood free glutamic acid content during the infusion of the various amino acid preparations the following procedure was used. A sample of venous blood was obtained and allowed to clot in a centrifuge tube; the unit dose of amino acid solution was then infused through the same needle. The rate of infusion was intentionally varied from patient to patient, but for any one subject the rate was maintained constant either until the patient became nauseated or vomited or until the infusion was ended. At the appearance of the above-mentioned symptoms or at the end of the infusion a blood sample was withdrawn from the opposite arm.

The solutions given to the subjects in Group B were prepared in two concentrations, the lower containing 5.05 Gm. of glutamic acid plus 2.8 Gm. of $NaHCO_3$ per 400 ml. of physiologic saline, and the higher containing twice this amount of glutamic acid and bicarbonate. In order to obtain information regarding the effect of the method of sterilization of these products on the incidence of toxic symptoms, some subjects of Group B received solutions sterilized by autoclaving at 15 pounds pressure for fifteen minutes. Other subjects were given solutions identical in all other respects except that they were sterilized by filtration through a Seitz filter.

In the series of studies in which solutions of partially neutralized glutamic acid (Group B) were infused at increasing rates, the procedure was essentially the same as outlined for Group A, except that the rate of infusion was increased every ten to fifteen minutes. A sample of blood was withdrawn for determination of the free glutamic acid content just before each increase in rate. This procedure was continued either until the patient became nauseated or vomited or until the infusion was completd.

The subjects in Group C received intravenously Preparation Vb to which was added 5.05 Gm. of glutamic acid per 400 ml. of solution. The procedure for administering this solution was the same as for the other amino acid preparations (Group A).

To reduce the psychologic factors which might influence emesis, the intravenous injections of the amino acids or glutamic acid were given as part of the routine therapy. Since other patients in the ward were getting intravenous therapy it was not considered an unusual procedure. No leading questions were asked during the injections.

For the evaluation of the free glutamic acid content of the various preparations used and of the serum of the patients, the microbiologic method of Dunn[7] was employed, using *Lactobacillus arabinosus*. Protein-free filtrates were prepared according to the method of Hier,[8] modified to a slight extent by immersing the tubes containing the filtrates of serum in boiling water for a few moments to remove the last traces of protein. Using this procedure, there was no precipitation of protein during the autoclaving of the media containing the test sample. The free glutamic acid content of the various amino acid preparations was determined directly on the product. They were not subjected to a preliminary hydrolysis before analysis.

RESULTS

Studies Involving Amino Acid Preparations.—In Table I is presented the free glutamic acid content of the various preparations used. The arrangement of the preparations is in decreasing order of glutamic acid content per unit dose of amino acid solution. Since, clinically, the amino acids are usually injected in the amounts contained in the units in which they are supplied, it was desirable to determine the free glutamic acid content in such units; Preparation

*Preparation VI was a 5 per cent protein hydrolysate in distilled water supplied in 1,000 c.c. units through the courtesy of the Baxter Laboratories, Glenview, Ill.

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II is an exception in that the solution supplied must be diluted before administration. Previous experience in this hospital[9] showed that the solution used was a satisfactory one if administered at a slow rate. All preparations did not have an equal nitrogen content. A number of different lots of each preparation were used to obtain the values listed in Table I. Two lots of Preparation V are listed; the first (Va) consisted of a batch which possessed the highest glutamic acid content of any of this type of mixture. In the second (Vb) a lower glutamic acid content was found and represents an amount closer to the average obtained for this preparation.

TABLE I. GLUTAMIC ACID CONTENT OF AMINO ACID PREPARATIONS

PREPARATION	AMINO ACID CONTENT (GM. PER CENT)	CONCENTRATION OF AMINO ACID SOLUTION INFUSED (GM. PER CENT)	VOLUME INFUSED (ML.)	GLUTAMIC ACID CONTENT OF INFUSED SOLUTION (GM.)
Preparation II	15	7.5	400	5.05
Preparation I	10	10	500	4.09
Preparation VI	5	5	1,000	2.65
Preparation V				
a	10	10	500	1.16
b	10	10	500	0.25
Preparation III	8	8	500	0.0

A summary of the information obtained by infusing Preparation II is listed in Table II. This preparation contained the highest glutamic acid content (5.05 Gm. per 400 c.c.) of those studied. It can be observed that high serum free glutamic acid levels were reached. Nausea and vomiting was the usual reaction if the glutamic acid content of the blood exceeded 15.9 mg. per 100 milliliters. The fact that marked variations in individual tolerance occurs is evident in that one subject did not become nauseated even though he had a serum free glutamic acid level of 42.6 mg. per 100 milliliters.

TABLE II. THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING
(PREPARATION II, 5.05 GM. GLUTAMIC ACID PER 400 C.C. OF 7.5% SOLUTION)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
	BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
Preparation II				
	4.1	10.6	3.1	0
	5.0	14.1	2.9	0
	3.9	15.9	4.5	0
	3.6	33.0	6.2	Nausea
	3.5	39.0	9.1	Nausea
	3.5	39.0	14.0	Vomited
	4.9	42.6	4.5	0
	4.0	43.0	10.2	Nausea
	2.8	43.2	5.7	Vomited
	--	85.0	3.7	Vomited

In Table III are summarized the results obtained by infusing Preparation I (4.09 Gm. glutamic acid per 500 c.c.). All subjects who had blood free glutamic acid levels above 16 mg. per cent became ill. One subject vomited at a lower level (10.9 mg. per 100 ml.). In general, serum free glutamic acid values were not as high after the administration of this preparation as with the previous one.

TABLE III. THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION I, 4.09 GM. GLUTAMIC ACID PER 500 C.C.)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
	BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
Preparation I				
	3.7	4.8	7.1	0
	4.0	7.3	6.1	0
	7.4	8.2	5.3	0
	4.2	8.8	6.2	0
	6.2	10.7	8.1	0
	4.1	10.8	10.2	0
	4.8	10.9	10.7	Vomited
	--	13.8	7.7	0
	6.6	16.2	8.6	Vomited
	3.9	22.5	5.5	Vomited
	8.3	23.2	6.5	Vomited
	4.4	23.9	9.4	Vomited
	8.0	28.7	5.5	Vomited

Preparation VI (Table IV), an enzymatic digest of bovine blood proteins, had a lower free glutamic acid content (2.65 Gm. per 1,000 c.c.) than the previous two preparations and this preparation could be administered at more rapid rates without increasing the toxic symptoms. One subject became nauseated at the rather low serum free glutamic acid level of 9.7 mg. per 100 ml., and one subject did not become ill in spite of the high blood level of 22.6 mg. per 100 milliliters.

TABLE IV. THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION VI, 2.65 GM. GLUTAMIC ACID PER 1,000 C.C.)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
	BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
Preparation VI				
	2.1	4.5	14.7	0
	2.0	6.7	20.2	0
	2.8	6.7	12.5	0
	1.7	7.8	20.0	0
	2.3	9.7	31.2	Nausea
	4.5	9.8	13.3	0
	4.4	10.3	14.5	0
	6.2	13.9	18.2	Vomited
	4.9	16.9	33.3	Vomited
	3.6	22.6	25.6	0

Table V summarizes the data obtained using the preparations containing none or small amounts (1.10 to 0.25 Gm.) of glutamic acid. The serum free glutamic acid values obtained following the infusion of 500 ml. of an amino acid mixture containing no glutamic acid is of particular interest. In spite of the exceedingly rapid rate of administration (50 and 62 ml. per minute), the two subjects showed no toxic symptoms and no appreciable increase in the serum free glutamic acid content. With Preparations Va and Vb attempts were made to produce elevated blood levels by increasing the rate of infusion. It was possible to increase the rate of injection of Preparation Va to such an extent (62.5

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ml. per minute) that nausea occurred. At the same time the blood free glutamic acid level rose to 12 to 15 mg. per 100 milliliters. With Preparation Vb, in only one case of the twelve studied was toxicity noted, but there were no rates of administration which were comparable with the faster infusions given with Preparation Va.

TABLE V. THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION Va, 1.10 GM. GLUTAMIC ACID; PREPARATION Vb, 0.25 GM., AND PREPARATION III, 0.0 GM.)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
	BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
Preparation Va				
	2.2	4.9	12.5	0
	2.7	7.0	14.3	0
	4.7	7.2	20.0	0
	6.2	7.5	13.5	0
	5.0	8.3	15.6	0
	4.1	9.1	16.4	0
	4.0	9.3	35.6	0
	5.5	12.3	62.5	Slight nausea
	--	14.7	62.5	Slight nausea
	4.9	15.7	71.4	Nausea
Preparation Vb				
	4.0	3.0	8.3	0
	3.1	3.7	9.1	0
	3.8	3.8	16.6	0
	--	4.8	12.5	0
	3.5	4.9	6.3	0
	5.2	5.8	9.6	0
	4.9	6.3	8.8	0
	1.9	6.3	7.1	0
	4.8	6.6	7.1	0
	3.9	7.4	21.7	Vomited
	3.9	7.9	23.8	0
	5.4	9.1	6.0	0
Preparation III				
	5.0	5.9	50.0	0
	5.4	6.3	62.5	0

The data presented in Tables I to V are summarized in Fig. 1. There is good correlation between the serum free glutamic levels and the per cent of subjects who either became nauseated or vomited. Without regard to the preparation used, when the blood free glutamic acid concentration reached 12 to 15 mg. per 100 ml. about one-half of the subjects became nauseated or vomited. Above these levels the incidence of ill effects was correspondingly greater.

The subjects of Group C received infusions consisting of Preparation Vb plus 5.05 Gm. of glutamic acid per 400 ml. of solution (Table VI). The glutamic acid content of the fortified preparation was thus made equal to an acid digest of casein (Preparation II). In this series there was a decidedly greater frequency of toxic symptoms as compared with this same mixture before glutamic acid was added (Table V). With Preparation Vb alone, the incidence of toxic symptoms was 8.3 per cent; with the addition of glutamic acid the incidence rose to 55 per cent. All of the subjects who experienced ill effects had serum free

glutamic acid levels equal to or above the 12 to 15 mg. per 100 ml. range. It is noteworthy that three of the subjects who had high serum glutamic acid values did not exhibit any gastrointestinal symptoms (Table VI).

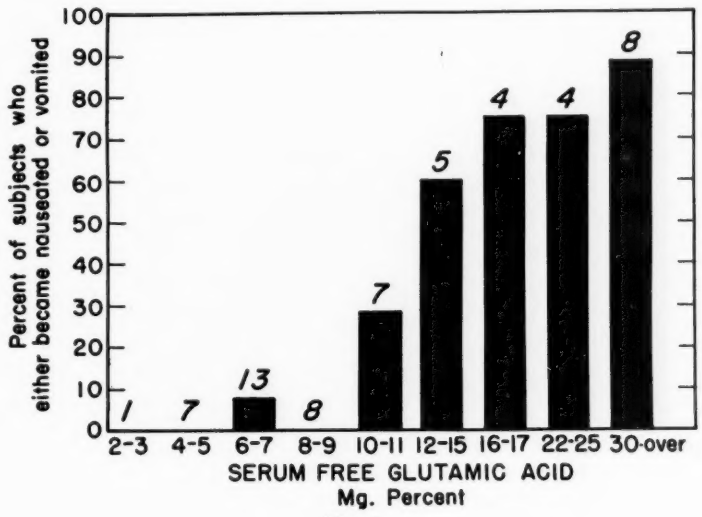

Fig. 1.

TABLE VI. THE EFFECT OF ADDING GLUTAMIC ACID TO PREPARATION VB ON THE OCCURRENCE OF NAUSEA AND VOMITING

(5.05 GM. OF GLUTAMIC ACID ADDED TO 400 C.C. OF PREPARATION VB AND STERILIZED BY AUTOCLAVING)

SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
2.0	9.0	5.8	0
4.5	9.4	9.3	0
--	11.8	8.0	Nausea
3.4	11.9	8.0	Nausea
3.0	18.0	12.0	Nausea
4.3	19.8	9.3	0
5.9	22.0	10.6	0
5.8	27.6	10.6	0
5.2	29.0	10.6	Nausea
4.8	30.0	9.3	Nausea
7.5	35.0	8.6	Nausea

Studies Involving Only Glutamic Acid Solutions.—In the next series of studies an attempt was made to determine whether there was a free glutamic acid level of the serum at which the occurrence of nausea and vomiting could be expected (Table VII, upper half). The first eight subjects received infusions of solutions containing 5.05 Gm. of partially neutralized glutamic acid per 400 ml. of solution; the next nine subjects received infusions of a solution containing 10.1 Gm. of glutamic acid per 400 milliliters. The level of serum glutamic acid reached was dependent on the concentration of the solution infused and the rate of injection. With the lower concentration none of the eight subjects who received the amino acid exhibited ill effects. Only two of these subjects had

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serum free glutamic acid levels exceeding 12 mg. per 100 milliliters. Nine subjects received the stronger solution, five of whom became nauseated or vomited. It should be noted that with the stronger solution higher serum free glutamic acid levels were quickly obtained. Forty-five per cent of the subjects who had levels of glutamic acid above 12 mg. per 100 ml. became nauseated or vomited.

TABLE VII. THE RELATIONSHIP BETWEEN SERUM FREE GLUTAMIC ACID LEVELS AND THE OCCURRENCE OF NAUSEA AND VOMITING AFTER GIVING GLUTAMIC SOLUTION INTRAVENOUSLY

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (C.C./MIN.)	REACTION
	BEFORE INFUSION (MG. PER CENT)	AFTER INFUSION (MG. PER CENT)		
Sterilized by Autoclaving				
Glutamic acid 5.05 Gm./400 c.c.	6.8	5.5	5.3	0
	3.3	6.4	11.0	0
	5.7	7.1	4.0	0
	5.3	8.0	4.6	0
	8.4	8.8	5.3	0
	5.4	11.5	10.0	0
	7.5	16.6	8.0	0
	3.5	35.0	40.0	0
Glutamic acid 10.1 Gm./400 c.c.	4.3	20.0	9.3	Vomited
	3.1	22.7	8.0	Vomited
	5.7	25.5	9.3	Vomited
	2.7	29.3	10.0	0
	4.0	31.0	12.0	0
	2.0	37.2	10.0	Vomited
	2.1	39.9	15.0	0
	5.9	41.8	10.0	0
	4.1	53.0	9.3	Vomited
Sterilized by Filtering				
Glutamic acid 5.05 Gm./400 c.c.	3.9	12.0	8.0	0
	3.6	15.1	10.6	0
	3.5	18.0	8.0	0
	3.3	22.6	8.0	Nausea and vomiting
	5.0	26.4	10.6	0
	4.1	27.3	8.0	Nausea
Glutamic acid 10.1 Gm./400 c.c.	4.4	8.5	6.6	Vomited
	3.3	14.9	8.0	Nausea and vomiting
	2.7	19.3	6.6	Vomited
	2.6	22.4	6.6	Vomited
	4.8	24.0	8.0	Vomited
	6.7	29.7	6.6	Vomited
	2.7	30.9	10.0	Nausea
	2.9	60.0	10.0	Vomited

Since many of the commercial protein digests are sterilized by filtration rather than autoclaving and since heat promotes the conversion of glutamic acid to pyrrolidone carboxylic acid, it was decided to repeat these studies using only glutamic acid solutions which had been sterilized by filtration, employing both the lower and higher doses (Table VII, lower half). When the lower dose was administered, two of the six subjects tested became nauseated or vomited; with the higher doses all of the eight subjects tested became ill. One subject vomited but had a blood glutamic acid level of only 7.5 mg. per 100 milliliters.

DISCUSSION

Until the present investigation the quantitative relationship of glutamic acid to the production of anorexia, nausea, and vomiting following the intravenous administration of casein hydrolysates was based on indirect evidence. Previous studies in this hospital indicated that no significant ill effects attended the giving of amino acid preparations orally; however, a depression of appetite, nausea, or vomiting frequently occurred with or immediately after infusions of casein digests.[10] These ill effects could be reduced to a large extent by reducing the rate of infusion. It was next observed that mixtures of the ten essential amino acids could be given intravenously at very rapid rates without producing ill effects.[6, 9, 11] Both Eckhardt and Davidson[12] and Weinstein[13] have administered intravenously to patients amino acid preparations containing reduced amounts of glutamic and aspartic acids and they have reported few toxic symptoms even with rapid rates of infusion. In the case of the dog[1, 2, 3, 4] (and later confirmed in man[5]), it was noted that the addition of either glutamic or aspartic acids to parenterally administered amino acid mixtures reduced the tolerance of these mixtures. Additional evidence indicated that there was a marked correlation between the content of these two dicarboxylic amino acids in amino acid preparations and the tendency of the solutions to provoke nausea and emesis in man. On the other hand, Elman[14] has reported the intravenous injections of an unneutralized glutamic acid solution (1,000 ml. of solution contained 30 Gm.) into three patients without the occurrence of any ill effects. No reference was made by this investigator to the rate of infusion, or to the glutamic acid level which resulted. His interpretation, apparently based upon that study, that intravenously injected glutamic acid is not related to the production of nausea and vomiting, is at variance with our experience.

Hoffman and co-workers[15] reported that if the blood amino acid nitrogen following the administration of an acid hydrolysate of casein exceeds 10 mg. per cent, nausea and vomiting may occur. This finding has been confirmed previously in this laboratory[6] for the same preparation; however, using preparations which were either free of or very low in glutamic acid it was possible to obtain markedly elevated blood amino acid nitrogen values without ill effects. At that time it was suggested that the blood glutamic acid probably was more closely related to the nausea and vomiting than the amino acid nitrogen content. Kade[16] has suggested that following the intravenous administration of preparations rich in glutamic acid the blood level of glutamic acid rises faster than the amino acid nitrogen content of the same blood. Friedberg and Greenberg[17] have reported that glutamic acid is slowly cleared from the blood of rats after intravenous administration.

In this study the determination of the free glutamic acid content of serum was accomplished by using a microbiologic procedure employing *Lactobacillus arabinosus* as the test organism. This method has been shown to give reliable values when protein hydrolysates were investigated.[7] Recently it was stated by Prescott and Waelsch[18] that the microbiologic method for glutamic acid is not applicable in the presence of glutamine. By their chemical method it was found

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that free glutamic acid of serum or plasma varied from 0.6 to 1.7 mg. per 100 ml., and the "apparent glutamine" content ranged from 8 to 12 mg. per 100 milliliters. Harper,[19] using a modified type of microbiologic method, more recently found glutamic acid and glutamine values in plasma similar to those given by the chemical method. The major portion of the preinfusion values for serum free glutamic acid reported in the present study would tend to show that a portion of the serum glutamine is being included in the value, because they are above the 0.6 to 1.7 mg. range. Since they are almost all below the 8 to 12 mg. range, not all the glutamine is being determined as free glutamic acid.

There appears to be some quantitative difference in the tolerance of glutamic acid when administered alone and when mixed with other amino acids. When 5.05 Gm. of glutamic acid were given intravenously as a solution containing only this amino acid, 14 per cent* (two of the fourteen studied) of the subjects either became nauseated or vomited. When a similar amount of glutamic acid was given intravenously in a mixture of amino acids (Preparation II), 60 per cent* (six of the ten tested) of the subjects showed ill effects. On the other hand, when the concentration of glutamic acid was increased to 10.1 Gm. and only this partially neutralized amino acid was given intravenously, 76 per cent (thirteen of the seventeen patients) of the subjects became nauseated or vomited. Thus, the incidence of nausea and vomiting following the intravenous administration of glutamic acid incorporated in an amino acid mixture is greater than that when an equivalent amount of partially neutralized glutamic acid is given alone. Both aspartic acid[2] and phenylalanine[20] have been reported to increase the toxicity of intravenously administered amino acid. dl-Methionine also has been listed as an amino acid which is poorly tolerated when given intravenously to the dog.[21] The amino acid preparations which are low in glutamic acid are also generally low in aspartic acid. Phenylalanine and methionine are probably not offenders in this manner, at least not without the dicarboxylic amino acids being present. Preparations III and V contained these acids and were well tolerated even at rapid rates. The possibility of other amino acids increasing the effect of glutamic acid in the production of nausea and vomiting following its intravenous administration should be further investigated.

In attempting to evaluate the data concerning the relative toxicity of the autoclaved as compared with the filtered solutions of glutamic acid, it was noted that 71 per cent* (ten of the fourteen cases) of the subjects that received the latter preparation became nauseated or vomited, while only 30 per cent* (five of the seventeen subjects) became ill after receiving the autoclaved material. Thus, the filtered material would appear to be slightly more toxic than the autoclaved. Olcott[22] reported that if glutamic acid were autoclaved at a temperature of 125° C. and in an acid media, pH 3.3 for four hours, 92 per cent of the amino acid would be converted to pyrrolidone carboxylic acid. The possibility of this occurring during the relatively short period of autoclaving used in the present

*Since the difference is greater than two times the standard error of the mean, these differences are considered significant.

study might be an explanation for the apparent difference in emetic effect. The likelihood of the pyrrolidone carboxylic acid assaying like glutamic acid (e.g., supporting the growth of the *Lactobacillus arabinosus*) while having an emetic action different from glutamic acid was investigated; in this case a high serum glutamic acid level might be attained without nausea and vomiting. Lyman found that this microorganism cannot utilize pyrrolidone carboxylic acid.[23] In the present study* it was found that pyrrolidone carboxylic acid neither stimulated nor inhibited the bacterial growth in that assay of glutamic acid when present in one-third to four times the concentration of glutamic acid. If much glutamic acid were converted to pyrrolidone carboxylic acid during the short period of autoclaving, then the concentration of free glutamic acid in the autoclaved material should decrease, since the pyrrolidone carboxylic acid does not support growth of the microorganism. This did not occur. Hence, it seems unlikely that pyrrolidone carboxylic acid could account for the apparent difference in toxicity of the filtered and autoclaved glutamic acid solutions.

SUMMARY AND CONCLUSIONS

The free glutamic acid content of five different types of amino acid preparations was estimated using microbiologic methods. They are, in decreasing order of glutamic acid content: an acid hydrolysate of casein fortified with tryptophane, an enzymatic hydrolysate of casein, an enzymatic digest of beef blood proteins, a mixture of amino acids produced by the recombination and fortification of fractions of a casein digest, and a mixture of the ten "essential" amino acids plus glycine. The occurrence of nausea and vomiting following the administration of the various preparations in a series of fifty-seven human subjects was found to parallel the free glutamic acid content of the mixture given.

Until the present investigation, only indirect evidence was obtained concerning the quantitative relationship of glutamic acid and the production of nausea and vomiting following its intravenous administration. A direct relationship between the free glutamic acid of the serum and the occurrence of toxic effects following the administration of amino acid preparations was found in this investigation. When the serum free glutamic acid level reached 12 to 15 mg. per 100 ml., nausea and vomiting occurred in more than half of the subjects.

Solutions containing only partially neutralized glutamic acid were administered intravenously to thirty-one individuals and estimates of the free glutamic acid content of the serum were made. A lower percentage of these patients became ill at elevated glutamic acid levels than in the group that received glutamic acid containing amino acid mixtures. This is interpreted as showing that the toxicity of glutamic acid may be potentiated by other amino acids.

A study of the effect of autoclaving and of sterilization by filtration upon the toxicity of glutamic acid solutions indicated that the filtered material was slightly more toxic. This difference could not be attributed to the presence of pyrrolidone carboxylic acid.

*These data are not included in the present report.

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REFERENCES

1. Madden, S. C., Woods, R. R., Shull, F. W., Remington, J. H., and Whipple, G. H.: Tolerance to Amino Acid Mixtures and Casein Digests Given Intravenously; Glutamic Acid Responsible for the Reactions, J. Exper. Med. **81**: 439, 1945.
2. Unna, K., and Howe, E. E.: Toxic Effects of Glutamic and Aspartic Acid, Federation Proc. **4**: 138, 1945.
3. Sokalchuk, A., Ellis, B. A., Wester, M. R., Weston, K., Greisheimer, E. M., and Oppenheimer, M. J.: Comparative Effects of Protein Hydrolysates and Amino Acid Mixtures on Intestinal Motility and Blood Sugar Levels After Rapid Intravenous Injections, Gastroenterology **10**: 831, 1948.
4. Lasichak, A. G., and Levey, S.: Glutamic Acid and Vomiting in Dogs: Its Administration Into the Portal System and Extremity Veins, Proc. Soc. Exper. Biol. & Med. **70**: 74, 1949.
5. Smyth, C. J., Levey, S., and Lasichak, A. G.: The Relationship of Glutamic and Aspartic Acids to the Production of Nausea and Vomiting in Man, Am. J. M. Sc. **214**: 281, 1948.
6. Smyth, C. J., Lasichak, A. G., and Levey, S.: The Effect of the Rate of Administration of Amino Acid Preparations and the Blood Amino Acid Nitrogen Level On the Production of Nausea and Vomiting, J. LAB. & CLIN. MED. **32**: 889, 1947.
7. Dunn, M. X., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C.: Investigations of Amino Acids, Peptides, and Proteins, XVII. The Determination of Glutamic Acid in Protein Hydrolysates by A Microbiological Method, J. Biol. Chem. **155**: 591, 1944.
8. Hier, S. W., and Bergeim, O.: The Microbiological Determination of Free Leucine, Isoleucine, Valine, and Threonine in Dog Plasma, J. Biol. Chem. **161**: 717, 1946.
9. Smyth, C. J., Levey, S., and Lasichak, A. G.: The Effects of the Rate of Administration of Amino Acid Preparations on Urinary Wastage of Amino Acid Nitrogen in Man, J. Clin. Investigation **27**: 412, 1948.
10. Smyth, C. J., Lasichak, A. G., and Levey, S.: The Effect of Orally and Intravenously Administered Amino Acid Mixtures on Voluntary Food Consumption in Normal Man, J. Clin. Investigation **26**: 439, 1947.
11. Werner, S. C.: The Use of Mixtures of Pure Amino Acids in Surgical Nutrition, Ann. Surg. **126**: 169, 1947.
12. Eckhardt, R. D., and Davidson, C. S.: Urinary Excretion of Amino Acids Following the Rapid Injection of a Solution of Amino Acids in Man, J. Clin. Investigation **27**: 727, 1948.
13. Weinstein, J. J.: Intravenous, Subcutaneous and Rapid Intramuscular Infusions of Protein Hydrolysates, Surg., Gynec. & Obst. **87**: 93, 1948.
14. Elman, R.: The Intravenous Use of Protein and Protein Hydrolysates, Ann. New York Acad. Sc. **47**: 345, 1946.
15. Hoffman, W. S., Kozoll, D. D., and Osgood, B.: Blood Chemical Changes Following Intravenous Administration of a Casein Hydrolysate to Human Subjects, Proc. Soc. Exper. Biol. & Med. **51**: 137, 1946.
16. Kade, C. F.: Personal communication.
17. Friedberg, F., and Greenberg, D. M.: Partition of Intravenously Administered Amino Acids in Blood and Tissues, J. Biol. Chem. **168**: 411, 1947.
18. Prescott, B. A., and Waelsch, H.: Free and Combined Glutamic Acid in Human Blood Plasma and Serum, J. Biol. Chem. **167**: 855, 1947.
19. Harper, H. A.: The Microbiological Determination of Glutamine in Human Plasma, Arch. Biochem. **15**: 433, 1947.
20. Madden, S. C., Bassett, S. H., Remington, J. H., Martin, F. J. C., Woods, R. R., and Shull, F. W.: Amino Acids in Therapy of Disease; Parenteral and Oral Administrations Compared, Surg., Gynec. & Obst. **82**: 131, 1946.
21. Howe, E. E., Unna, K., Richards, G., and Seeler, A. O.: Comparative Tolerance to Mixtures of Natural and Racemic Amino Acids on Intravenous Infusion in the Dog, J. Biol. Chem. **162**: 395, 1946.
22. Olcott, H. S.: A Method for the Determination of Glutamic Acid in Proteins, J. Biol. Chem. **153**: 71, 1944.
23. Lyman, C. M., Kuiken, K. A., Blotter, L., and Hale, F.: The Microbiological Determination of Amino Acids. II. Glutamic Acid, J. Biol. Chem. **157**: 395, 1945.

CARBONIC ANHYDRASE ACTIVITY IN SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PERNICIOUS ANEMIA

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CARBONIC anhydrase, an enzyme first described by Meldrum and Roughton[1] and Stadie and O'Brien,[2] is present in the erythrocytes of homothermic animals and in the gills of some poikilothermic ones.[3] It plays an important role in the transport and exchange of CO_2. No studies have been reported previously on the presence of carbonic anhydrase in sickle cell anemia, although this enzyme has been implicated in the sickling phenomenon by Tomlinson and Jacob.[4] These authors found that washing erythrocytes from patients with sickle cell anemia or sicklemia twenty times in normal saline almost always removes their ability to assume sickled forms in normal saline. They suggested that the multiple washings remove some substance, possibly carbonic anhydrase, the presence of which is necessary for the phenomenon of sickling. Additional evidence for this hypothesis was presented by the demonstration that certain carbonic anhydrase inhibitors, such as zinc acetate and sodium cyanide, in concentrations of 1:1000, when added to oxygenated blood, prohibit sickle cell formation; however, other carbonic anhydrase inhibitors, namely sulfanilamide and ammonium thiocyanate, gave inconsistent results.

In the present studies the relationship between carbonic anhydrase and the sickling phenomenon was investigated by the following means. (1) The carbonic anhydrase content of the blood of patients with sickle cell anemia and with sickle cell trait was compared with that of normal individuals. Three cases of pernicious anemia were included for comparison. (2) Erythrocytes from patients with the sickling tendency were washed repeatedly in normal saline, until sickling no longer occurred; the red blood cell suspension was then made up to its original volume with water, and again tested for the presence of carbonic anhydrase. The saline washings also were tested for the presence of carbonic anhydrase. (3) The susceptible erythrocytes were washed repeatedly until sickling no longer occurred, and a concentrated solution of human carbonic anhydrase was added to determine whether such addition restored the ability of the cells to sickle.

METHODS

Carbonic anhydrase determinations were made by the colorimetric method of Philpot and Philpot,[5] and enzyme units were calculated according to this method. Each determination was made in triplicate. Oxalated blood was used throughout. Appropriate dilutions

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were made of whole blood, except in those experiments in which washed cells were used. Concentrated carbonic anhydrase preparations were made by treating the washed and hemolyzed red blood cells with a mixture of alcohol and chloroform and centrifuging off the precipitate. The carbonic anhydrase activity of the supernatant fluid was about fifty times that of the whole blood.

TABLE I. CARBONIC ANHYDRASE CONTENT OF BLOOD OF NORMAL INDIVIDUALS AND THOSE WITH SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PERNICIOUS ANEMIA

1. CARBONIC ANHYDRASE CONTENT OF BLOOD OF NORMAL INDIVIDUALS

DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C.MM.)	HEMOGLOBIN (GM./100 C.C.)	CARBONIC ANHYDRASE (ENZYME UNITS/C.MM.)
1/7	M. J. P.	4.57	14.4	1,220
1/7	G. M.	5.09	16.7	1,240
1/7	F. S.	4.42	12.7	960
3/4	B. L.	4.92	17.0	846
3/16	J. B.	5.82	16.5	814
3/16	P. T.	5.7	16.5	1,380
4/14	L. J.	4.2	12.7	1,280
4/18	M. S.	4.5	13.0	1,026
4/27	S.	4.14	12.7	1,140
Average		4.81	14.68	1,100

2. CARBONIC ANHYDRASE CONTENT OF BLOOD OF PATIENTS WITH SICKLE CELL ANEMIA

DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C.MM.)	HEMOGLOBIN (GM./100 C.C.)	CARBONIC ANHYDRASE (ENZYME UNITS/C.MM.)
3/23	A. R.	2.41	6.7	670
3/31	W. S.	4.17	9.4	692
3/31	M. H. F.	3.48	9.4	780
4/4	P. P.	2.87	10.5	732
4/7	W. B.	3.0	10.4	814
4/27	O. W.	2.27	7.0	940
Average		3.03	8.9	771
Per cent of normal		62	60	71

3. CARBONIC ANHYDRASE CONTENT OF BLOOD OF INDIVIDUALS WITH SICKLE CELL TRAIT

DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C.MM.)	HEMOGLOBIN (GM./100 C.C.)	CARBONIC ANHYDRASE (ENZYME UNITS/C.MM.)
1/7	L. P.	3.58	11.3	1,120
1/7	C. J.	4.51	12.1	1,020
1/7	J. C.	4.08	12.7	1,020
1/7	N. B.	4.55	14.6	980
4/4	G. M.	4.1	13.2	1,060
4/14	L. G.	3.66	9.4	1,260
4/27	E. C.	4.68	14.0	960
Average		4.76	12.4	1,060
Per cent of normal		86	84	96

4. CARBONIC ANHYDRASE CONTENT OF BLOOD OF INDIVIDUALS WITH PERNICIOUS ANEMIA

DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C.MM.)	HEMOGLOBIN (GM./100 C.C.)	CARBONIC ANHYDRASE (ENZYME UNITS/C.MM.)
1/4	T. B. L.	2.21	9.1	880
1/4	S.	2.03	9.3	1,020
3/16	T.	1.23	3.3	680
Average		1.82	7.23	1,000
Per cent of normal		38	49	91

Sickling of cells was estimated by examining sealed wet smears of blood after incubation at room temperature for twenty-four hours. The erythrocytes were washed twenty times with normal saline according to the technique described by Tomlinson and Jacob.[4] In certain instances carbonic anhydrase was added to whole blood and to cells washed five, ten, fifteen, and twenty times. The amount of sickling after such treatment was compared with that of untreated cells.

RESULTS

In Table I is given the carbonic anhydrase content of the blood of individuals with sickle cell anemia and sickle cell trait as compared with normal individuals and three persons with pernicious anemia. In Table II these data are summarized in the form of ratios of carbonic anhydrase to red blood cell count and hemoglobin content. These ratios indicate that the carbonic anhydrase content of red blood cells with the sickling capacity is as high as or

TABLE II. RATIO OF BLOOD CARBONIC ANHYDRASE TO RED BLOOD CELLS AND HEMOGLOBIN OF NORMAL INDIVIDUALS AND THOSE WITH SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PERNICIOUS ANEMIA

NUMBER OF PATIENTS	DIAGNOSIS	RATIO OF AVERAGE CARBONIC ANHYDRASE TO AVERAGE R.B.C. COUNT	RATIO OF AVERAGE CARBONIC ANHYDRASE TO AVERAGE HEMOGLOBIN CONCENTRATION
9	Normal	1:228	1:74
6	Sickle cell anemia	1:254	1:87
7	Sickle cell trait	1:223	1:85
3	Pernicious anemia	1:549	1:138

slightly higher than that of normal red blood cells. In pernicious anemia, red blood cells contain much larger quantities of carbonic anhydrase; although only three cases of pernicious anemia were studied, the difference appears striking.

Table III summarizes the data obtained by determining the carbonic anhydrase content of cells with the sickling capacity washed twenty times in isotonic saline until sickling no longer occurred. Ninety per cent of the carbonic anhydrase content of the original whole blood is present in these washed cells. The saline washings contain only a trace of carbonic anhydrase; one determination of the carbonic anhydrase activity of the plasma of these washed cells is noted. Part of the carbonic anhydrase lost by washing is apparently contained in the plasma, where it doubtless appears because of hemolysis.

TABLE III. CARBONIC ANHYDRASE CONTENT OF RED BLOOD CELLS AFTER WASHING UNTIL SICKLING CAPACITY IS REMOVED

PATIENT	DIAGNOSIS	CARBONIC ANHYDRASE WHOLE BLOOD (ENZYME UNITS/C.MM.)	CARBONIC ANHYDRASE WASHED CELLS (ENZYME UNITS/C.MM.)	CARBONIC ANHYDRASE SALINE WASHINGS	CARBONIC ANHYDRASE CONTENT OF PLASMA (ENZYME UNITS/C.MM.)
P. P.	Sickle cell anemia	732	708	—	
O. W.	Sickle cell anemia	940	840	Trace	
E. C.	Sickle cell trait	960	840	Trace	76
Average		877	796		

Table IV illustrates the results of adding concentrated carbonic anhydrase solution to cells from which the sickling capacity has been removed by repeated washing in isotonic saline. Such addition of carbonic anhydrase does not restore the sickling capacity of the cells.

TABLE IV. EFFECT OF CARBONIC ANHYDRASE ON THE ABILITY OF SUSCEPTIBLE CELLS TO SICKLE AFTER MULTIPLE WASHINGS WITH ISOTONIC SOLUTION OF SODIUM CHLORIDE

DATE	PATIENT	DIAGNOSIS	NUMBER OF WASHINGS	PER CENT SICKLING OF UNTREATED CELLS	PER CENT SICKLING AFTER CONCENTRATED CARBONIC ANHYDRASE ADDED	PER CENT SICKLING AFTER ADDITION OF CARBONIC ANHYDRASE DILUTED 1:10 WITH 0.85% NaCl
2/11/49	W. B.	Sickle cell anemia	0	9.6		16
			5	0		0.6
			10	0		6.0
			15	0		0
			20	0		0
2/10/49	M. F.	Sickle cell anemia	0	82.8		85.2
			5	80		78
			10	60.8		0
			15	25.6		37.4
			20	28.2		17.4
2/10/49	A. A.	Sickle cell anemia	0	75.4		80.4
			5	0		0
			10	0		0
			15	0		0
			20	0		0
2/18/49	R. A.	Sickle cell anemia	0	92.2		91
			5	62		77.8
			10	12.2		6.4
			15	13.6		Hemolyzed
			20	13.0		Hemolyzed
3/ 7/49	A. R.	Sickle cell anemia	0	Hemolyzed	15.6	8.4
			5	10	Hemolyzed	15.8
			10	7.2	3.9	5.4
			15	13.6	10	10.8
			20	12	30.2	9.8

DISCUSSION

Our results indicate that the loss of sickling capacity of red blood cells from patients with sickle cell anemia or sickle cell trait is not associated with a significant loss of carbonic anhydrase. After the completion of these experiments there appeared in *Blood* an editorial by Vallee and Altschule[6] referring to a series of papers in press on carbonic anhydrase in anemia. In this editorial and in an abstract recently published by Vallee[7] a striking difference is drawn between the carbonic anhydrase content of red blood cells from patients with pernicious anemia and those from patients with secondary anemias and from normal individuals. Our findings indicate that blood from sickle cell anemia and sickle cell trait is similar to that of secondary anemia in this respect. In contrast, our three cases of pernicious anemia have twice the ratio of carbonic anhydrase to red blood cells and hemoglobin compared with the normal values.

SUMMARY

1. The carbonic anhydrase content of red blood cells exhibiting the sickling phenomenon corresponds to that of normal red blood cells.

2. Washing red blood cells of individuals with sickle cell anemia or sickle cell trait with isotonic saline until sickling no longer occurs results in no significant loss of carbonic anhydrase activity.

3. Addition of carbonic anhydrase to washed cells from which the sickling capacity has been removed does not restore this capacity.

4. Carbonic anhydrase, under conditions described, has no effect on the sickling phenomenon.

5. Confirmatory evidence is presented that in pernicious anemia unit values of carbonic anhydrase in respect to red blood cell count and hemoglobin are about twice those of normal individuals.

REFERENCES

1. Meldrum, N. U., and Roughton, F. J. W.: Carbonic Anhydrase. Its Preparation and Properties, J. Physiol. **80:** 113-142, 1933.
2. Stadie, W. C., and O'Brien, H.: The Catalysis of the Hydration of Carbon Dioxide and Dehydration of Carbonic Acid by an Enzyme Isolated From Red Blood Cells, J. Biol. Chem. **103:** 521-529, 1933.
3. Ferguson, J. K. W., Lewis, L., and Smith, J.: Carbonic Anhydrase in Marine Invertebrates, Am. J. Physiol. **119:** 308-309, 1937.
4. Tomlinson, W. J., and Jacob, J. E.: Studies of Sickle Cell Formation in Normal Saline, Plasma and Sera With Carbonic Anhydrase Inhibition, J. LAB. & CLIN. MED. **30:** 107-111, 1945.
5. Philpot, F. J., and Philpot, J.: A Modified Colorimetric Estimation of Carbonic Anhydrase, Biochem. J. **302:** 2191, 1936.
6. Vallee, B. L., and Altschule, M. D.: Trace Metals in Blood With Particular Reference to Zinc and Carbonic Anhydrase, Blood **4:** 398-402, 1949.
7. Vallee, B. L.: Zinc and Carbonic Anhydrase Content of Red Cells in Normals and in Pernicious Anemia, J. Clin. Investigation **27:** 559, 1948.

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CLINICAL STUDIES ON THIOMERIN, A NEW MERCURIAL DIURETIC

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SEVERAL effective mercurial diuretics are available and are widely used at the present time. The introduction of a new diuretic can be justified only if it is less toxic or more effective, or if it is more convenient to use. This report concerns itself with the results of a study of a new mercurial diuretic whose toxicity and efficiency have been compared with those of several of the preparations in common usage. The new material is the disodium salt of N(γ-carboxymethyl mercaptomercuri β-methoxy) propyl camphoramic acid (Thiomerin).* It has the same organic-mercurial structure as Mercuzanthin, with the theophylline replaced by sodium mercaptoacetate. Thiomerin is a white amorphous powder which is supplied in vials containing 1.4 Gm. of the compound. The introduction of 10 c.c. of sterile water into the vial results in a solution containing the equivalent of 0.040 Gm. of mercury per cubic centimeter at a pH of about 7.5.

METHODS

The method of study was similar to that employed by DeGraff.[1] Patients in congestive heart failure and with considerable edema were digitalized and kept at bed rest on a relatively salt-poor diet (approximately 3 grams of sodium chloride daily). There was no limitation of fluid intake. Each patient's weight was recorded daily at the bedside until there was no further weight loss over the course of several days. At this point the diuretic was administered. Patients who were too ill to be weighed or whose weight might have been affected by variation in dietary intake, vomiting, decrease in hyperthyroid state, or by factors other than edema were not included in this study. Each patient's urine was examined for abnormalities before and after the administration of the diuretic. Injections of Thiomerin were given subcutaneously, except in two instances when it was administered intravenously. The injections of Mercuzanthin and of Salyrgan-Theophylline were given intramuscularly.

RESULTS

The diuretic effect of Thiomerin in seventy trials on thirty-six patients is indicated in Fig. 1. There was diuresis resulting in weight losses ranging from 2 to 17½ pounds. This range of weight loss was roughly proportional to the amount of edema present. In all the Thiomerin trials (in which weight loss was studied) the average weight loss was 6 pounds and the median weight loss was 5 pounds, while somewhat less than 50 per cent of injections resulted in a weight loss between 3 and 4 pounds. The diuretic effect usually appeared within several hours after the injection and lasted for twenty-four hours or more. The duration of action varied. Usually it was over by the end of twenty-four hours, although in several patients it lasted for three days. Variation in duration of

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*Thiomerin has the following structural formula: C $(CH_3)_2C(COONa)$ (CH_3) $CH_2CH_2CHCONHCH_2CH(OCH_3)CH_2HgSCH_2COONa$.

action sometimes occurred in the same patient from one injection to the next. In no instance was there any evidence of any nephrotoxic effect. From these results it can be seen that Thiomerin is an effective diuretic.

The diuretic effect of Thiomerin was next compared with that of two widely used mercurial diuretics: Mercuzanthin and Salyrgan-Theophylline. Approximately the same amount of mercury (0.04 Gm.) is present in 1 c.c. of all these preparations.

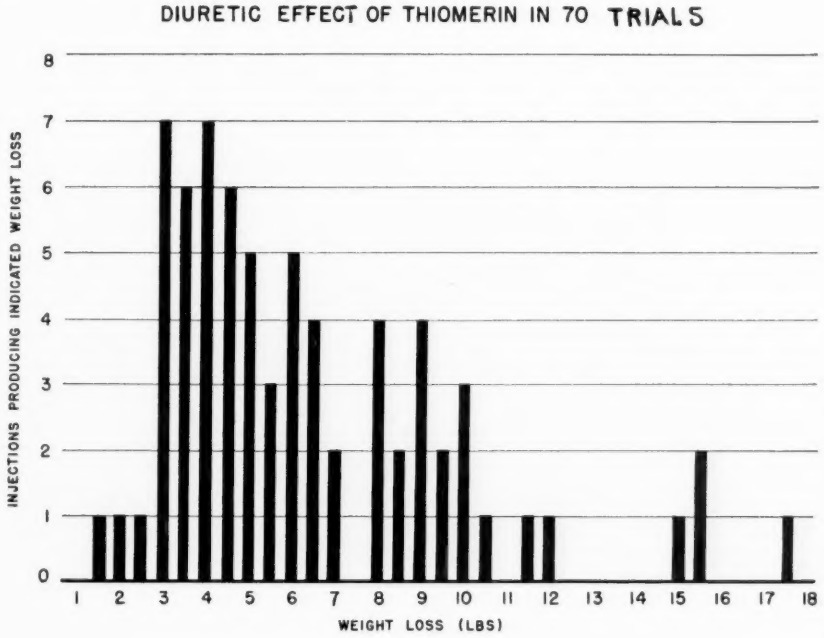

Fig. 1.

As shown in Fig. 2, nineteen comparisons of the diuretic activity of Thiomerin and Salyrgan-Theophylline were made in thirteen patients. In seven instances (37 per cent) the diuretic effect was the same. In nine instances (47 per cent) Thiomerin produced a greater diuresis, and in three instances (16 per cent) Salyrgan-Theophyllin had a greater effect. The duration of diuretic action was also noted for the two drugs. In six instances the Thiomerin-induced diuresis was more prolonged; in two instances a more sustained effect was induced by Salyrgan-Theophylline. In Case 44, Thiomerin-induced diuresis in two trials was more prolonged and greater than that obtained with Salyrgan-Theophylline. This was true despite the fact that the Thiomerin was given intravenously on one occasion and subcutaneously on the next.

The relative diuretic action of Thiomerin and Mercuzanthin was observed in ten comparisons on eight patients. In five of these instances the diuresis obtained was the same; in three it was greater with Thiomerin, and in two it was greater with Mercuzanthin. It also was noted that in three instances Thiomerin-induced diuresis was slower and more prolonged.

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Toxicity.—Urinalyses before and after the administration of Thiomerin did not reveal evidence of renal toxicity. This was true in a patient with chronic glomerulonephritis as well as in the patients with heart disease of other etiology.

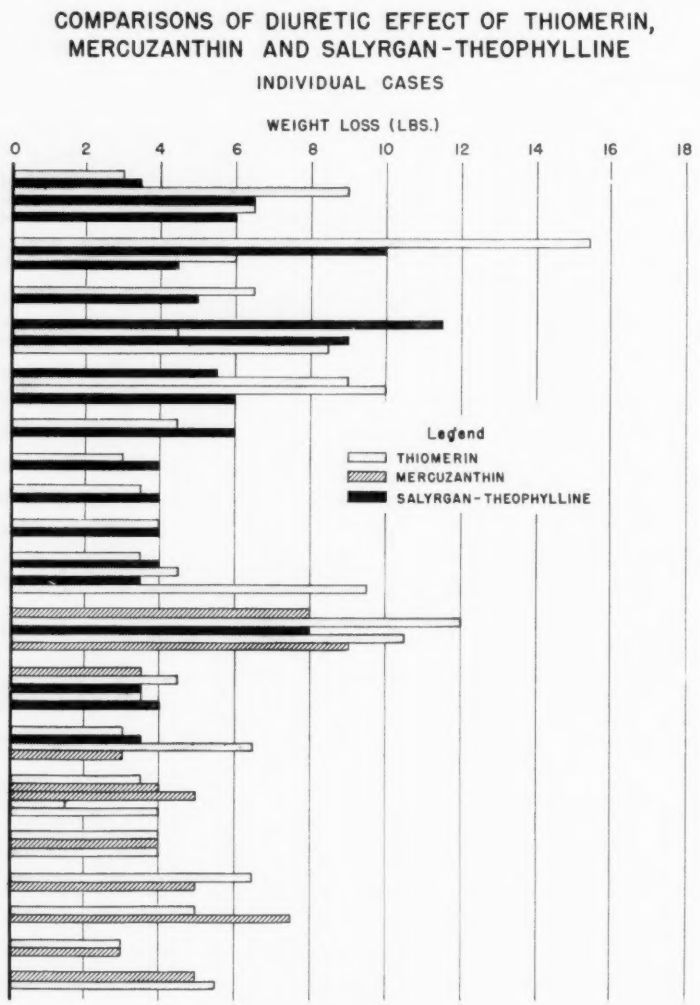

Fig. 2.

Since Thiomerin is given subcutaneously, local effects were watched for. Immediate reactions were minimal. They consisted of slight burning or stinging at the site in 41 per cent of the injections. A small number of patients also had some aching or local soreness lasting a few minutes to a few days. Because a certain number of nodules or infiltrations were found to follow use of the drug, it was decided to study this long-term phenomenon more carefully in another group of patients. While the early product, no longer being manufactured, caused appreciable indurations in 12 per cent of injections, the drug as

it is now being prepared gave rise to such infiltrations only four times (3.3 per cent) in a total of 121 injections. These areas, which were similar after both products, were situated subcutaneously and could usually not be outlined clearly since they faded into the surrounding tissue. They were about ¼ in. to 1½ in. in diameter, were up to ⅓ in. in depth, were occasionally attached to the skin, and were only rarely tender. They cleared up gradually in a period of two to five months. Biopsies done on four of these infiltrations showed them to consist of areas of aseptic fat necrosis. In this last series of 121 injections there were also six instances (5 per cent) of local infiltrations so small that they would have been missed had they not been searched for carefully.

DISCUSSION

With the extensive use of mercurial diuretics, a number of reports have appeared which emphasize fatal or near fatal reactions from their intravenous use.[2, 3, 4] The possibility of such toxic reactions has been present regardless of the mercurial preparation employed. It is due to direct action on the heart with the production of disturbances in conduction and rhythm[5, 6] and in working capacity.[7] A recent study in animals of the comparative toxicity of the commonly used mercurial diuretics and of Thiomerin has shown the latter to be 160 to 200 times less toxic to the heart.[8] No effect was seen by electrocardiogram in anesthetized cats after a dose of 4 c.c. of Thiomerin per kilogram, as compared with 0.1 c.c. per kilogram of other mercurial diuretics. If these results may be transposed to man, this would compare a dose of 240 c.c. of Thiomerin to one of 6 c.c. of Mercuzanthin, Salyrgan-Theophylline, or Mercuhydrin. Thus the danger of sudden cardiac death would seem to be practically eliminated.

Our results confirm the findings of Borg and Craig[9] and Hermann and coworkers[10, 11] as to the efficacy of Thiomerin as a diuretic for subcutaneous administration. The diuresis obtained by us following the subcutaneous injection of Thiomerin was comparable with that obtained with equivalent amounts of Mercuzanthin and Salyrgan-Theophylline given intramuscularly. In some patients it was a more effective diuretic. The effect was more sustained in a number of instances. This may be related to the site of administration, although in one patient the intravenous injection of Thiomerin produced more sustained diuresis than did an intramuscular injection of Salyrgan-Theophylline.

SUMMARY

1. The diuretic effectiveness of Thiomerin, a new mercurial compound which is reported to be less toxic to the heart when administered intravenously to cats, has been investigated in man.

2. Thiomerin was found to be an effective diuretic in seventy trials on thirty-six patients.

3. The diuresis obtained after the subcutaneous injection of 2 c.c. of Thiomerin was comparable with or greater than that noted after the intramuscular use of equivalent amounts of Mercuzanthin or Salyrgan-Theophylline in twenty-nine comparative studies on twenty-one patients. In some patients the diuresis with Thiomerin was more sustained.

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4. No systemic toxic effects were noted. Kidney damage, as judged by urine examinations, did not occur.

5. Local reactions following the subcutaneous use of Thiomerin were absent or insignificant 96.7 per cent of the time. Three and three tenths per cent of injections were followed by annoying but seemingly harmless subcutaneous infiltrations of long duration.

REFERENCES

1. DeGraff, A. C., Nadler, J. E., and Batterman, R. C.: A Study of the Diuretic Effect of Mercupurin in Man, Am. J. M. Sc. **191**: 526-538, 1936.
2. DeGraff, A. C., and Nadler, J. E.: Review of Toxic Manifestations of Mercurial Diuretics in Man, J. A. M. A. **119**: 1006-1011, 1942.
3. Wexler, J., and Ellis, L. B.: Toxic Reactions to the Intravenous Injection of Mercurial Diuretics, Am. Heart J. **27**: 86-95, 1944.
4. Kaufman, R. E.: Immediate Fatalities After Intravenous Mercurial Diuretics, Ann. Int. Med. **28**: 1040-1047, 1948.
5. Volini, I. F., Levitt, R. O., and Martin, R. J.: Studies on Mercurial Diuresis; Sudden Death Following Intravenous Injection: Report of 3 Cases With Electrocardiographic Studies in 2, J. A. M. A. **128**: 12-17, 1945.
6. DeGraff, A. C., and Lehman, R. A.: Acute Toxicity of Mercurial Diuretics, J. A. M. A. **119**: 998-1101, 1942.
7. Long, W. K., and Farah, A.: Influences of Certain Sulfhydryl Compounds on Toxicity of Organic Mercurial Diuretics, J. Pharmacol. & Exper. Therap. **88**: 388-399, 1946.
8. Lehman, R. A.: Further Studies on the Acute Toxicity of Mercurial Diuretics, Proc. Soc. Exper. Bio. & Med. **64**: 428-433, 1947.
9. Borg, J. F., and Craig, D. M.: Experiences With a Mercurial Diuretic for Subcutaneous Use, J. Lab. & Clin. Med. **33**: 1635-1636, 1948.
10. Hermann, G. R., Chriss, J. W., Hejtmancik, M. R., and Sims, P. M.: Treatment of Myocardial Failure, Texas State J. Med. **45**: 79-82, 1949.
11. Hermann, G. R., Chriss, J. W., Hejtmancik, M. R., and Sims, P. M.: Modern Treatment of Edema, With a Report of Relatively Low Toxicity and Effectiveness of Thiomerin a New Mercurial Diuretic That Is Well Tolerated Subcutaneously, Am. Pract. 3: 393-399, 1949.

LIMITATIONS AND MERITS OF A SINGLE SERUM SAMPLE ANALYSIS IN THE DIFFERENTIAL DIAGNOSIS OF JAUNDICE

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INTRODUCTION

PREVIOUS experiences in the employment of multiple biochemical procedures to assay liver function and to assist in determining the cause of jaundice have been reported from this clinic.[1, 2] The differential diagnosis of jaundice is a problem that can be solved only by the exercise of sound clinical judgment at the patient's bedside. Laboratory studies are at best only auxiliary aids to this problem. Until we possess specific biologic tests for the recognition of virus hepatitis, the detection of this disorder depends chiefly upon inferences drawn from the history of the illness, the physical examination, and the laboratory evidence of liver dysfunction. Since we lack a nonoperative roentgenologic method of visualizing the extrahepatic biliary tract in the jaundiced patient, the diagnosis of obstruction due to calculi, to tumors, or to stricture must similarly be based upon inferences from such data.

In 1945, Schwimmer, Klotz, Drekter, and McGavack[3] described their extensive experience with a fasting blood sample in the differential diagnosis of jaundice. They pointed out the many practical advantages of this procedure. The single specimen group of tests employed by these investigators included the icteric index, the van den Bergh reaction, the cephalin-cholesterol flocculation test, the serum alkaline phosphatase value, the total and esterified serum cholesterol, and the total serum proteins together with the albumin and globulin fraction. The application of these tests to a wide variety of hepatic disorders by Schwimmer and associates revealed that the "composite test" yielded results that were as delicate, as accurate, and as informative as those derived from the more elaborate tests of liver function.

Shay and Siplet[4] stated that a "minimum yet adequate liver function study" may be made in jaundice with the following tests: (1) quantitative van den Bergh, (2) serum cholesterol partition, (3) serum alkaline phosphatase, (4) thymol turbidity, and (5) cephalin-cholesterol flocculation. They illustrated the merits of these tests when applied serially to individual cases. More recently, Popper and Steigmann[5] have evaluated the merits of these tests, as well as others in current usage, in 285 patients. The initial series of liver function tests was taken into consideration in that study.

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The present study was made to test the accuracy with which a group of tests performed on a *single* fasting serum sample can assist in the differential diagnosis in a jaundiced patient. It is not our contention that the analysis of a single specimen of serum from a jaundiced patient should be entirely adequate. Serial observations are certainly indicated and were, in fact, carried out in the majority of our patients. These often included tests other than those to be discussed in this paper.

MATERIALS AND METHODS

In the selection of cases for the present study only instances of regurgitation jaundice have been chosen for analysis. Instances of retention jaundice usually have other distinguishing features and seldom present the diagnostic problems that regurgitation jaundice does. One hundred forty-seven patients observed at the University of Minnesota Hospital were included. The following criteria were employed in the selection.

1. Presence of jaundice. The study has been limited to instances in which the prompt direct reacting component (1 min. value) of the serum bilirubin was greater than 0.4 mg. per 100 c.c. and the total bilirubin greater than 2.0 mg. per 100 cubic centimeters.

2. Available data. Evaluation has been limited to instances in which the following determinations were made on a *single* sample of serum secured in the fasting state: bilirubin, total and esterified cholesterol, alkaline phosphatase, thymol turbidity, and cephalin-cholesterol flocculation.

3. Proof of diagnosis. Evaluation has been limited to patients in whom the diagnosis was established by operation, necropsy, or biopsy except in the cases of virus hepatitis where a clinical diagnosis was accepted despite lack of biopsy data. A critical appraisal of the available clinical material was made and the selection was restricted to instances in which the course of the illness was believed to establish the diagnosis beyond reasonable doubt.

Of the 147 patients, seventy-seven had extrahepatic biliary obstruction and seventy suffered from parenchymal liver disease. The former group included:

1. Twenty-eight cases of carcinoma obstructing the bile ducts.
2. Thirty-nine cases of common duct calculus.
3. Ten cases of stricture of the bile ducts.

In each instance the diagnosis was verified by operation or by necropsy.

The group of cases of parenchymal jaundice included:

1. Twenty-six cases of cirrhosis.
2. Thirty-one cases of virus hepatitis.
3. Thirteen cases of liver disease due to miscellaneous causes.

The methods employed are listed in Table I. In three cases (see Table VI) and in one case (Table VII) the thymol turbidity was performed according to the colorimetric adaptation of Ducci.[12] The upper limit however is the same as by the Maclagan method.[13]

TABLE I. BIOCHEMICAL PROCEDURES EMPLOYED

PROCEDURE	METHOD	NORMAL VALUE
1. Serum bilirubin	Malloy and Evelyn[6] as modified by Ducci and Watson[7]	1′ (prompt direct) 0.2 mg. / 100 c.c. (total, direct plus indirect) 1.0 mg. / 100 c.c.
2. Cephalin-cholesterol flocculation	Hanger[8]	A reading greater than 1+ at 24 hr. may be considered abnormal
3. Thymol turbidity	Maclagan[9]	0 – 4 units
4. Serum cholesterol (total)	Schoenheimer and Sperry[10]	180 – 220 mg. per 100 c.c.
5. Serum cholesterol (esterified fraction)	Schoenheimer and Sperry[10]	50 – 65% of total
6. Alkaline phosphatase	Bodansky[11]	1 – 4 units per 100 c.c.

RESULTS

The values presented in Table II indicate the anticipated results if one were to assume that these procedures would always yield results of differential diagnostic value. The values given are admittedly arbitrary. It is well known that certain cases of diffuse liver disease exhibit functional changes that are very similar to those produced by mechanical obstruction of the extrahepatic bile ducts. Such instances may be considered examples of cholangiolar dysfunction. However, the majority of cases of hepatitis and cirrhosis might be expected to exhibit hepatocellular dysfunction and to conform therefore to anticipated results insofar as these tests are concerned.

The actual values obtained in the different types of jaundice are given in Tables III to VIII. The cases have been arranged in order of the increasing duration of jaundice insofar as the duration could be ascertained from the history. The numbers in italics in these tables indicate instances in which the actual results fail to conform with those listed in Table II as anticipated results. These data have been tabulated in Table IX.

TABLE II. ANTICIPATED RESULTS IN BIOCHEMICAL TESTS EMPLOYED IN PRESENT STUDY

TYPE OF LIVER DISEASE	CEPHALIN-CHOLESTEROL (24 HR. READING)	THYMOL TURBIDITY (MACLAGAN)	TOTAL SERUM CHOLESTEROL	CHOLESTEROL ESTER (PERCENTAGE)	ALKALINE PHOSPHATASE (BODANSKY)
Extrahepatic obstruction	1+ or below	0 – 4 units	Above 225 mg. per 100 c.c.	Above 50%	Above 10 units
Parenchymal liver disease	Above 1+	Above 4 units	Below 225 mg. per 100 c.c.	Below 50%	Below 10 units

Bilirubin.—In Tables III through VIII, the relationship of the one-minute serum bilirubin to the total has been computed as percentage. The data for the serum bilirubin are presented in graphic form in Fig. 1 where the cases are arranged in order of increasing duration of jaundice. No consistent pattern of behavior is discernable.

Serum Cholesterol.—An analysis of Table IX reveals that the total serum cholesterol was below 225 mg. per 100 c.c. in twenty instances in the seventy-seven cases of extrahepatic biliary obstruction. Furthermore, the cholesterol esters constituted less than 50 per cent of the total in thirty-four cases in this group. The cholesterol ester fraction was below 50 per cent in twenty-one of the twenty-eight cases in the carcinoma group.

The cholesterol levels failed to correspond with the anticipated results in twenty-two of the seventy cases of parenchymal liver disease. The thirty-one cases of hepatitis included in these seventy cases represent a more homogeneous group. Even so, eleven of these thirty-one patients had cholesterol levels greater than 225 mg. per 100 c.c. at the time the multiple tests were performed. Nine of these eleven patients had ester fractions which comprised more than 50 per cent of the total cholesterol.

TABLE III. RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO CARCINOMA

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
1	M	77	2 days	1.0	2.2	45	*174*	92	53	0	0	2	32	Operation
2	F	83	6 days	4.2	7.4	57	288	46	*16*	*3+*	*4+*	*6*	25	Necropsy
3	F	81	7 days	17.5	30.7	57	360	50	*14*	Tr.	1+	2	50	Necropsy
4	M	71	10 days	9.9	17.0	58	354	103	*29*	0	0	2	18	Operation
5	M	56	12 days	9.2	15.2	61	*198*	115	58	*2+*	*3+*	1	31	Necropsy
6	M	60	14 days	3.8	6.2	61	358	218	61	0	1+	3	*6*	Operation
7	M	40	14 days	9.9	18.1	55	234	133	57	1+	1+	3	*6*	Operation
8	M	66	3 wk.	13.7	24.0	57	350	126	*36*	0	0	3	22	Operation
9	M	70	4 wk.	8.0	12.1	66	*114*	30	*26*	1+	1+	3	12	Necropsy
10	F	71	4 wk.	15.6	27.9	55	676	277	*41*	0	Tr.	3	69	Necropsy
11	F	60	4 wk.	14.5	25.4	57	594	184	*31*	0	Tr.	2	24	Operation
12	F	62	4 wk.	8.6	18.2	47	570	274	*48*	0	0	*9*	22	Operation
13	F	75	5 wk.	9.0	14.5	62	238	74	*31*	0	1+	2	13	Necropsy
14	M	63	6 wk.	9.9	17.5	57	236	125	53	0	1+	2	30	Operation
15	M	68	6 wk.	18.7	63.5	29	310	46	*15*	1+	1+	2	19	Operation
16	M	59	6 wk.	24.0	41.6	58	506	40	*8*	0	0	1	31	Necropsy
17	M	56	6 wk.	9.4	20.7	45	236	47	*20*	1+	1+	*5*	31	Necropsy
18	M	61	6 wk.	3.3	7.1	46	268	185	69	0	Tr.	2	12	Operation
19	M	74	7 wk.	17.0	31.7	57	290	107	*37*	0	0	1	30	Operation
20	M	65	7 wk.	16.8	31.0	54	640	214	*33*	0	0	2	18	Necropsy
21	F	67	7 wk.	12.8	22.6	57	510	56	*11*	0	0	1	32	Operation
22	M	72	8 wk.	1.9	3.6	53	*208*	125	60	1+	1+	2	35	Operation
23	F	62	8 wk.	15.3	26.0	59	360	83	*23*	1+	1+	3	13	Operation
24	F	60	4 mo.	14.8	28.3	52	296	15	*5*	1+	1+	2	20	Necropsy
25	M	76	4 mo.	15.8	28.8	55	*130*	32	*25*	Tr.	1+	3	15	Necropsy
26	F	72	6 mo.	17.7	31.8	56	720	346	*48*	1+	1+	*5*	44	Necropsy
27	F	56	11 mo.	8.7	14.5	60	362	98	*27*	0	0	3	27	Operation
28	M	38	?	10.2	17.1	60	370	100	*27*	0	0	4	*9*	Operation

TABLE IV. RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO COMMON DUCT STONE

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
29	F	77	2 days	1.0	2.4	42	240	154	64	0	0	2	*9*	Operation
30	F	68	2 days	4.7	8.2	57	*100*	29	*29*	1+	1+	2	*6*	Necropsy
31	F	73	2 days	1.0	2.1	48	328	216	66	1+	1+	2	25	Operation
32	F	73	3 days	2.5	4.9	51	*164*	82	50	0	Tr.	2	*5*	Operation
33	F	62	3 days	1.6	3.2	50	*194*	116	60	0	1+	2	24	Operation
34	M	67	4 days	7.0	11.4	61	*124*	41	*33*	1+	2+	1	*3*	Operation
35	F	41	4 days	2.1	3.9	54	272	169	62	0	0	2	35	Operation
36	M	81	5 days	1.8	3.3	55	234	140	60	0	0	2	18	Necropsy
37	F	67	5 days	1.3	2.8	46	250	175	70	0	0	2	26	Operation
38	F	36	7 days	8.0	14.1	57	240	84	*35*	*3+*	*4+*	2	14	Operation
39	M	64	7 days	1.2	2.2	54	*152*	107	71	0	0	1	15	Operation
40	M	73	8 days	2.9	4.8	60	*126*	79	63	1+	2+	1	*5*	Operation
41	M	74	14 days	8.4	12.9	65	328	167	51	0	0	2	*3*	Operation
42	F	31	14 days	7.8	11.8	66	338	98	*29*	0	0	2	15	Operation
43	F	37	3 wk.	0.9	2.6	35	*160*	107	67	0	0	2	*6*	Operation
44	F	54	3 wk.	5.4	9.7	56	230	62	*27*	0	Tr.	1	12	Operation
45	M	57	3 wk.	6.0	8.6	63	*192*	117	61	1+	1+	2	34	Operation
46	M	58	4 wk.	1.7	3.8	45	234	150	64	0	0	1	19	Operation
47	F	52	6 wk.	5.8	12.0	48	234	122	52	0	0	3	*6*	Operation
48	F	37	6 wk.	3.9	6.8	57	276	124	*45*	0	1+	4	15	Operation
49	M	51	6 wk.	2.3	3.9	59	260	156	60	0	0	1	15	Operation
50	M	74	8 wk.	2.3	3.9	59	260	187	72	0	0	1	60	Operation
51	F	72	10 wk.	1.1	2.1	52	262	141	54	0	Tr.	2	14	Operation
52	F	56	10 wk.	4.6	8.7	53	668	100	*15*	0	0	*5*	55	Operation
53	M	73	12 wk.	1.4	3.0	47	*112*	73	65	0	1+	1	14	Operation
54	M	58	12 wk.	3.1	6.5	48	346	197	57	0	1+	1	11	Operation
55	M	76	12 wk.	1.6	3.4	47	252	108	*43*	0	0	1	14	Operation
56	F	44	4 mo.	1.5	3.2	47	244	159	65	0	0	1	18	Operation
57	F	47	6 mo.	5.3	11.2	47	394	193	*49*	0	0	3	33	Operation
58	F	52	6 mo.	1.0	2.0	50	*210*	147	70	0	0	1	14	Operation
59	M	77	6 mo.	5.1	9.9	52	*138*	55	*40*	*2+*	*3+*	3	*9*	Operation
60	F	45	9 mo.	4.4	7.0	63	336	185	55	0	0	2	19	Operation
61	F	72	1 yr.	1.1	2.2	50	226	131	58	0	0	3	27	Operation
62	F	53	2 yr.	1.0	2.2	45	294	218	74	0	0	3	18	Operation
63	M	74	2½ yr.	1.0	2.8	36	312	218	70	0	0	2	27	Operation
64	F	63	?	1.5	2.7	56	300	200	66	0	0	1	27	Operation
65	M	72	?	7.3	11.6	63	300	132	*44*	0	0	1	*6*	Operation
66	M	71	?	.8	2.4	33	*122*	78	64	0	0	1	*9*	Operation
67	F	55	?	3.0	5.0	60	232	155	67	1+	1+	1	*8*	Operation

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TABLE V. RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO COMMON DUCT STRICTURE

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
68	F	56	3 days	3.4	5.4	63	*140*	94	67	0	1+	3	34	Operation
69	M	66	7 wk.	15.1	27.7	55	*212*	49	*23*	0	0	2	12	Operation
70	F	34	8 wk.	3.5	6.8	51	362	188	52	0	0	2	37	Operation
71	M	36	12 wk.	0.5	2.1	24	*212*	146	69	0	1+	1	20	Operation
72	M	31	12 wk.	15.2	24.6	62	868	434	51	0	0	1	20	Operation
73	F	30	4 mo.	2.1	4.1	51	294	150	51	0	0	3	23	Operation
74	F	67	1 yr.	6.3	10.5	60	258	72	*28*	0	0	3	24	Operation
75	F	42	1 yr.	5.5	10.2	54	920	488	53	1+	1+	*6*	50	Operation
76	F	49	7 yr.	2.4	4.4	55	228	146	64	0	0	1	*4*	Operation
77	F	55	8 yr.	4.2	6.9	61	380	213	56	0	0	1	20	Operation

TABLE VI. RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO CIRRHOSIS

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
78	F	62	2 wk.	13.8	21.8	63	*276*	19	7	3+	4+	18	*13*	Biopsy
79	M	63	4 wk.	6.4	10.0	64	124	53	43	*0*	*0*	*3*	3	Necropsy
80	M	30	4 wk.	1.3	4.0	32	156	31	20	2+	3+	*4*	3	Necropsy
81	M	70	6 wk.	1.6	2.4	67	146	76	*52*	2+	3+	7	3	Biopsy
82	M	42	10 wk.	0.8	2.4	33	204	126	*62*	*1+*	2+	*4*	2	Biopsy
83	M	59	12 wk.	2.1	3.9	54	170	76	45	3+	4+	24*	5	Necropsy
84	M	33	12 wk.	13.1	27.6	47	96	22	23	3+	4+	14	*17*	Biopsy
85	F	35	4 mo.	2.4	5.1	47	*384*	230	*60*	2+	2+	9	*26*	Biopsy
86	F	62	5 mo.	3.2	5.5	58	*244*	146	*60*	*Tr.*	*Tr.*	9*	9	Operation
87	M	54	6 mo.	3.8	15.9	24	*248*	114	46	3+	4+	10	*14*	Necropsy
88	F	12	9 mo.	4.2	7.7	55	126	68	*54*	3+	4+	21	*11*	Biopsy
89	M	32	11 mo.	3.4	7.0	49	112	60	*54*	3+	3+	19	9	Operation
90	M	30	1 yr.	13.9	26.5	53	126	32	25	3+	4+	*3*	*13*	Necropsy
91	M	58	1 yr.	1.2	2.7	44	138	88	*64*	2+	3+	*2*	3	Biopsy
92	F	44	2 yr.	2.1	6.4	33	*314*	176	*56*	*1+*	2+	9	6	Biopsy
93	F	42	3 yr.	7.0	11.4	61	*430*	181	42	*1+*	2+	9	*39*	Necropsy
94	F	51	3 yr.	1.2	2.4	50	*504*	328	*65*	2+	3+	7	*18*	Biopsy
95	F	47	3 yr.	1.5	2.8	54	176	113	*64*	*1+*	2+	*4*	*18*	Biopsy
96	F	20	3 yr.	4.1	7.9	52	178	105	*59*	3+	4+	16	*13*	Clinical data
97	M	61	6 yr.	1.5	3.5	43	*248*	154	*62*	*0*	*1+*	6	*12*	Biopsy
98	F	37	7 yr.	1.7	2.7	63	*420*	214	*51*	*0*	*0*	*3*	*63*	Operation
99	F	54	10 yr.	0.8	2.0	40	156	89	*57*	*1+*	2+	*1*	*28*	Biopsy
100	M	8	?	1.0	3.2	31	108	55	*51*	3+	4+	21	4	Operation
101	F	8	?	2.6	5.6	46	136	49	36	3+	4+	10	6	Peritoneoscopy
102	F	10	?	1.3	2.0	45	92	30	33	3+	4+	9	9	Biopsy
103	F	23	?	4.8	8.0	60	196	114	*58*	2+	2+	*3*	*19*	Necropsy

*By the method of Ducci.[12]

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TABLE VII. RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO HEPATITIS

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
104	M	74	1 day	12.9	21.7	59	140	27	19	3+	3+	*2*	*11*	Biopsy
105	M	20	2 days	5.2	8.4	62	218	61	28	3+	4+	15	4	Clinical data
106	F	26	3 days	6.1	10.7	59	206	82	40	2+	3+	*3*	4	Clinical data
107	F	29	3 days	1.3	2.4	54	184	103	*56*	3+	4+	12	8	Clinical data
108	M	56	3 days	2.9	5.6	52	*230*	135	*58*	*0*	*0*	*1*	8	Clinical data
109	M	50	3 days	4.6	7.7	60	170	85	*50*	3+	4+	9*	3	Clinical data
110	M	12	4 days	5.7	9.2	62	153	23	15	2+	3+	21	9	Clinical data
111	M	59	4 days	25.0	39.0	64	176	42	24	3+	4+	16	8	Clinical data
112	M	26	4 days	3.8	7.5	51	*242*	145	*60*	2+	3+	8	*10*	Clinical data
113	F	19	6 days	3.1	6.6	47	210	65	31	3+	4+	8	4	Clinical data
114	M	33	7 days	7.8	13.0	60	166	61	37	3+	4+	14	8	Clinical data
115	M	64	7 days	15.0	27.7	54	*226*	34	15	3+	4+	5	7	Clinical data
116	M	4	8 days	4.3	7.3	59	*244*	44	18	2+	3+	18	*13*	Clinical data
117	M	20	8 days	1.7	4.6	59	132	61	46	2+	3+	6	5	Clinical data
118	F	20	9 days	2.8	5.4	52	196	59	30	*1+*	2+	12	6	Clinical data
119	F	32	10 days	1.8	3.0	60	*252*	88	35	3+	4+	18	8	Clinical data
120	F	19	10 days	1.9	3.5	54	*286*	109	38	3+	3+	30	8	Clinical data
121	F	20	11 days	3.2	7.0	46	186	93	49	3+	4+	9	7	Clinical data
122	M	31	14 days	11.7	19.0	62	154	31	20	3+	4+	6	4	Clinical data
123	F	34	3 wk.	2.6	5.7	46	*280*	126	45	2+	2+	16	5	Clinical data
124	M	64	3 wk.	3.0	5.2	78	*490*	314	*64*	3+	4+	21	*15*	Biopsy
125	F	12	3 wk.	3.1	5.1	61	156	75	48	2+	3+	12	*13*	Clinical data
126	F	49	3 wk.	4.2	7.5	56	214	66	31	3+	4+	33	*12*	Clinical data
127	F	46	3 wk.	14.0	26.9	52	81	12	15	2+	3+	6	8	Necropsy
128	F	13	8 wk.	2.6	4.8	54	100	30	30	2+	3+	5	*30*	Biopsy
129	M	41	8 wk.	5.9	10.1	58	212	117	*55*	*0*	*0*	*1*	*16*	Clinical data
130	F	31	12 wk.	7.7	15.2	51	118	61	*52*	3+	4+	*4*	3	Necropsy
131	M	32	4 mo.	3.9	8.3	47	*244*	151	*62*	*1+*	*1+*	15	5	Biopsy
132	F	55	4 mo.	9.2	16.1	57	*262*	24	9	3+	4+	27	9	Necropsy
133	F	18	6 mo.	3.7	7.7	48	158	90	*57*	3+	4+	10	4	Clinical data
134	M	58	9 mo.	.9	2.1	43	*260*	169	*65*	*1+*	2+	12	8	Biopsy

*By the method of Ducci.[12]

TABLE VIII. RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO MISCELLANEOUS CAUSES

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN-CHOLESTEROL		THYMOL TURBIDITY	ALKALINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	1′/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
						CARDIAC JAUNDICE								
135	F	39	4 wk.	0.8	2.4	33	100	70	*70*	2+	3+	6	3	Necropsy
136	M	57	4 wk.	7.0	12.6	56	64	20	31	2+	3+	*3*	2	Necropsy
137	M	71	8 wk.	1.1	3.5	31	170	114	*67*	*1+*	2+	*1*	4	Clinical data
138	M	45	9 mo.	1.0	2.4	42	116	73	*63*	*1+*	*1+*	8	5	Clinical data
139	M	52	?	1.7	2.7	63	206	144	*70*	2+	3+	*3*	3	Clinical data
						LYMPHOBLASTOMA								
140	F	15	2 wk.	2.9	5.6	51	90	8	9	*1+*	2+	*2*	6	Necropsy
141	M	47	7 wk.	0.7	2.9	24	*370*	74	20	*0*	*Tr.*	*2*	*102*	Biopsy
						SEPTICEMIA								
142	M	1	7 wk.	3.3	7.4	45	126	59	47	*Tr.*	*1+*	*2*	*18*	Blood culture
143	M	70	?	4.3	6.6	65	90	42	47	*1+*	2+	*1*	*12*	Blood culture
						SULFA REACTION								
144	M	13	6 days	7.5	13.1	57	200	44	22	*1+*	*1+*	6	*15*	Necropsy
						AMYLOIDOSIS								
145	M	68	?	0.8	2.4	33	*226*	131	*58*	*0*	*0*	16	4	Necropsy
						PERNICIOUS ANEMIA								
146	M	73	6 mo.	0.6	2.5	24	152	96	*63*	*0*	*0*	*1*	1	Hematology
						FATTY METAMORPHOSIS								
147	F	26	?	4.8	8.7	55	34	4	12	*1+*	2+	*1*	7	Necropsy

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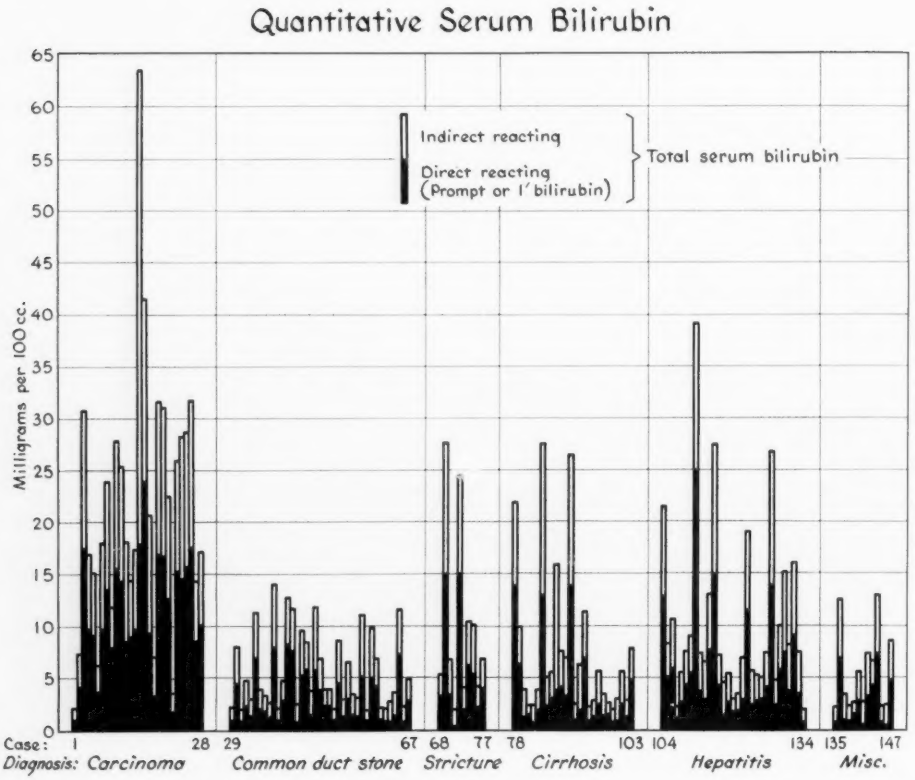

Fig. 1.

The cholesterol data are arranged in Fig. 2 in the order of the duration of jaundice.

Serum Alkaline Phosphatase.—The results of the individual tests appear in Tables III through VIII. The instances in which the anticipated values were not obtained are listed in Table IX. It may be seen that the results were quite similar to those obtained with the cholesterol determination. The data are presented graphically in Fig. 3 in the order of the duration of jaundice.

TABLE IX. NUMBER OF INSTANCES IN WHICH ACTUAL RESULTS FAILED TO CONFORM WITH ANTICIPATED RESULTS

TYPE OF DISEASE	NUMBER OF CASES	CEPHALIN-CHOLESTEROL	THYMOL TURBIDITY	SERUM CHOLESTEROL	CHOLESTEROL ESTER (PERCENTAGE)	ALKALINE PHOSPHATASE
Carcinoma	28	2	4	5	21	3
Common duct stone	39	2	1	12	11	12
Common duct stricture	10	0	1	3	2	1
Total	77	4	6	20	34	16
Cirrhosis	26	9	9	9	16	14
Hepatitis	31	5	6	11	10	8
Miscellaneous hepatic disease	13	10	9	2	6	4
Total	70	24	24	22	32	26

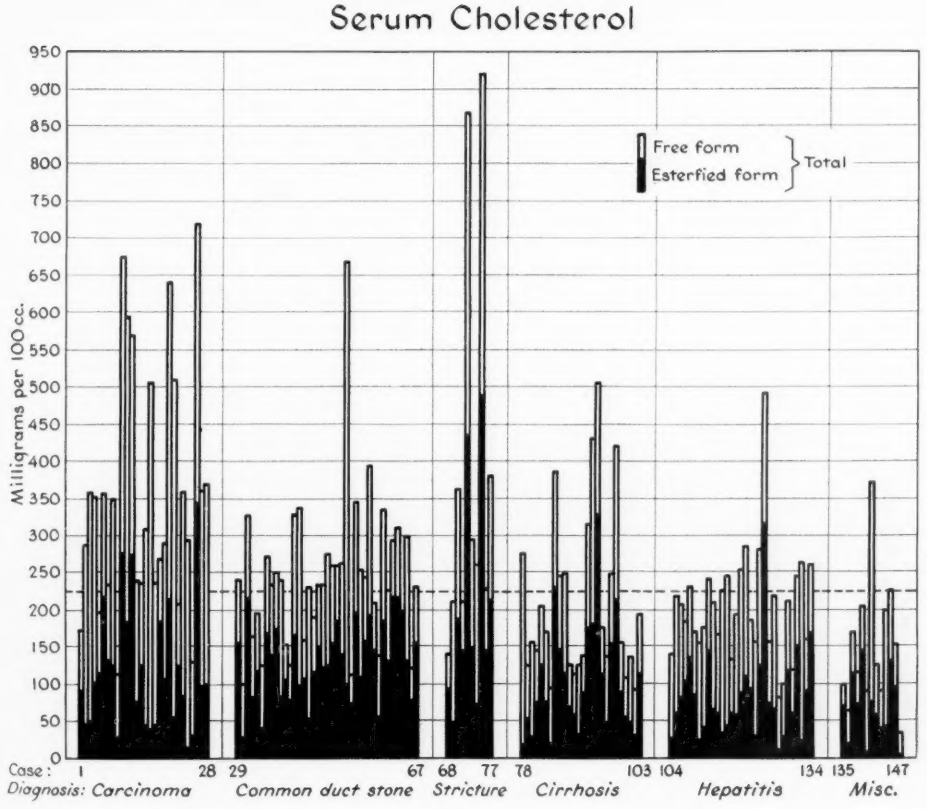
Serum Cholesterol
Free form
Esterfied form
Total
Milligrams per 100 cc.
950
900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Case: 1 28 29 67 68 77 78 103 104 134 135 147
Diagnosis: Carcinoma
Common duct stone
Stricture
Cirrhosis
Hepatitis
Misc.

Fig. 2.

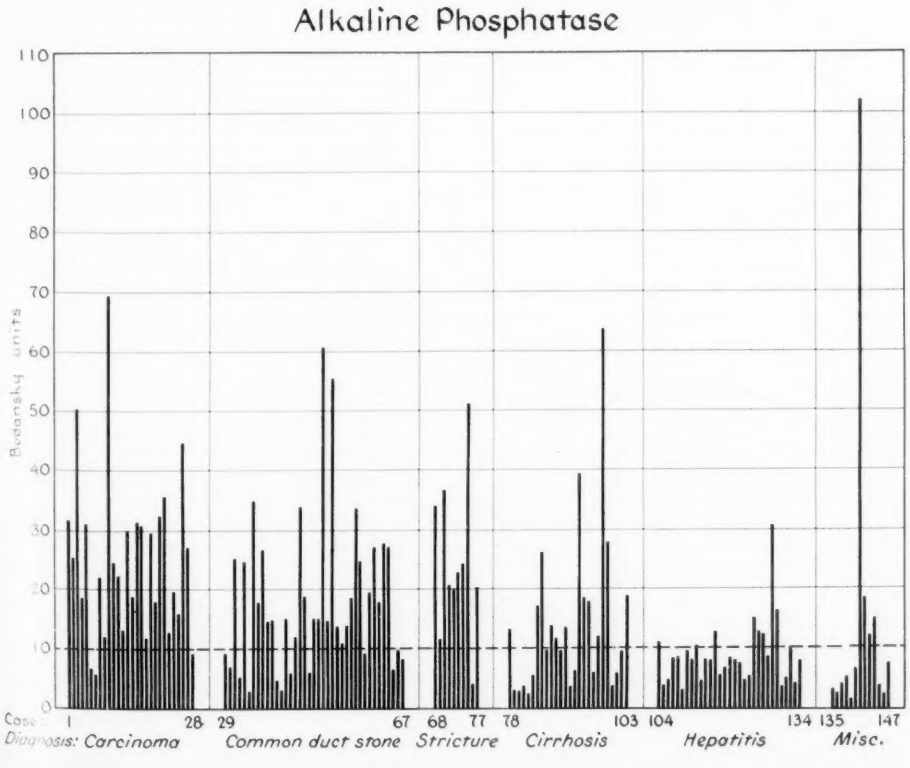
Alkaline Phosphatase
Bodansky units
110
100
90
80
70
60
50
40
30
20
10
0
1 28 29 67 68 77 78 103 104 134 135 147
Diagnosis: Carcinoma
Common duct stone
Stricture
Cirrhosis
Hepatitis
Misc.

Fig. 3.

Cephalin-Cholesterol Flocculation Test.—For the purpose of this analysis only the reading obtained at twenty-four hours has been considered although both values are presented in Tables III through VIII. The data for the twenty-four hour reading have been arranged according to the duration of

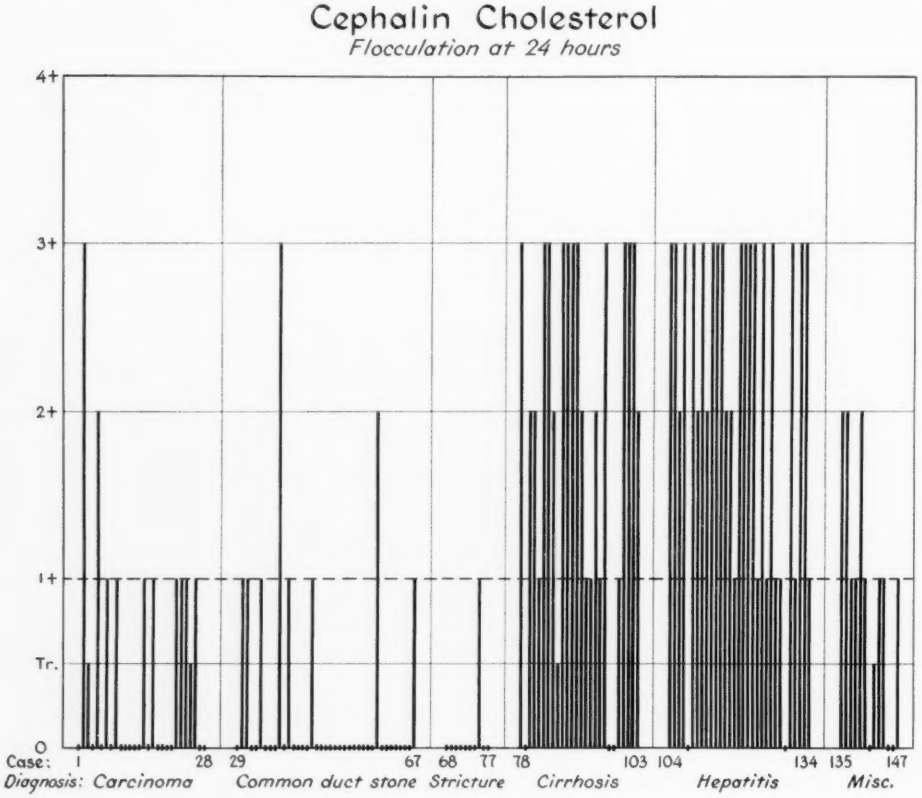

Fig. 4.

jaundice in Fig. 4. Among the seventy-seven cases of extrahepatic biliary obstruction there were four instances in which abnormal, and therefore presumably misleading, results were obtained.

The two patients in the carcinomatous obstructive group (Cases 2 and 5, Table III) both exhibited positive Hanger tests early in the course of their jaundice. In Case 2, the patient, an 83-year-old woman, had jaundice due to a carcinoma of the gall bladder. Subsequent cephalin-cholesterol flocculation tests also were positive repeatedly. The patient of Case 5, a 56-year-old man, proved to have a carcinoma of the pancreas with hepatic metastases. Serial determinations were not possible in this instance.

There were two proven instances of common duct stone obstruction in which the Hanger test was positive (Cases 38 and 50, Table IV). Jaundice associated with chills and fever had been present for seven days in Case 38; one week later the cephalin-cholesterol was only a trace at 24 hours, and though

jaundice had diminished, the other tests remained essentially unchanged. The presence of a positive test in Case 59 was not surprising since the patient had been jaundiced for six months.

There were twenty-four cases among the seventy examples of parenchymal liver disease in which the Hanger test was negative. This should occasion no surprise since the group is a heterogeneous one, and save for the thirty-one cases of hepatitis, uniform results would be unlikely.

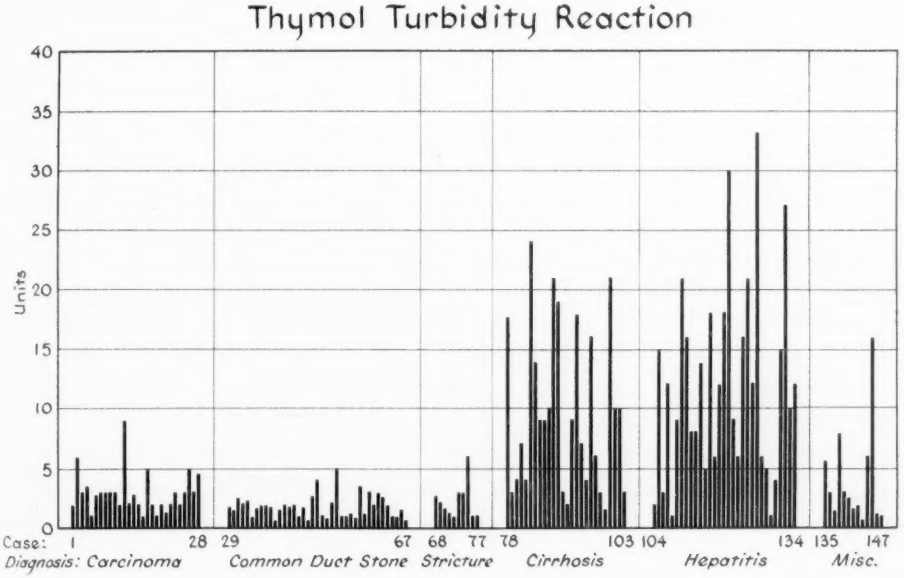

Fig. 5.

Thymol Turbidity.—The data relating to the thymol turbidity test, obtained from Tables III through VIII, are arranged graphically in Fig. 5 according to duration of jaundice. Inspection of Table IX and Fig. 5 reveals that the test gave positive results (values greater than 4 units) in four cases of carcinoma and one each in the patients with calculus or stricture.

Among the cases of cancerous jaundice with abnormal thymol turbidity tests, three patients (Cases 2, 17, and 26) had values just slightly above the level of 4 units which is taken as the dividing line. Case 12 revealed a value of 9 units. Only one patient in the group of calculus jaundice had a value above 4 units. This was Case 52 with 5 units. Similarly, one patient (Case 75, Table V) with a long-standing stricture of the bile duct revealed a thymol test of 6 units.

The seventy cases of parenchymal liver disease included twenty-four instances in which the thymol turbidity test was not elevated (Table IX).

Multiple Tests.—The principal purpose of this study was to determine the merit of multiple laboratory tests that can be performed on a single sample of serum. It is therefore necessary to analyze briefly how often certain combinations of these procedures gave misleading information. It is evident that the

quantitative van den Bergh reaction (Fig. 1) failed to give information of differential diagnostic value in this series of cases of regurgitation jaundice. The serum bilirubin will not, therefore, be considered further in relation to any combination.

The ester fraction of the serum cholesterol, when expressed as per cent of the total, failed to conform to the anticipated results in thirty-four cases in the extrahepatic obstructive group. In thirty-two cases in the parenchymal jaundice group the value of the cholesterol ester fraction was greater than 50 per cent and may, therefore, be considered unexpected. Because there appeared to be so much overlapping of values irrespective of what percentage level was chosen, this particular determination also will be eliminated from the present discussion of multiple tests.

Four procedures—the total cholesterol, the alkaline phosphatase, the cephalin-cholesterol flocculation, and the thymol turbidity—remain then for comparison. This comparison can best be made by examining the cases according to the established diagnoses.

A. Carcinoma: Results opposite to those anticipated were encountered in two cases; two of the four tests proved to be misleading. Case 2, in which both the cephalin-cholesterol and the thymol test were positive, has been discussed previously. The results were consistent on repeated examinations during the forty-five days that the patient survived. This was the only case in the group of seventy-seven surgical patients in which both the cephalin-cholesterol and the thymol turbidity were simultaneously positive and thereby misleading.

Case 5 also has been discussed previously. In that instance the cephalin-cholesterol flocculation value at 24 hours was 2 plus and the total cholesterol was only 198 mg. per 100 cubic centimeters. Only one set of determinations was made in this patient.

B. Common Duct Stone: In one patient (Case 59) in this group, three of the four tests under discussion were misleading. This elderly man had been jaundiced for six months. The total cholesterol was only 138 mg. per 100 c.c.; the cephalin-cholesterol reading was 2 plus, and the alkaline phosphatase value was 9 Bodansky units. Obstruction to bile outflow was presumably high grade since the total serum bilirubin at this time was 9.9 mg. per 100 cubic centimeters. The fecal urobilinogen was not determined, but the presence of increased urobilinogen in the urine indicated incomplete obstruction.

In six cases in this group, two of the four tests failed to conform to the expected results. In each of these six instances (Cases 30, 32, 34, 40, 43, and 66) the total cholesterol ranged from 100 to 164 mg. per 100 c.c. and the alkaline phosphatase was not elevated above 10 Bodansky units. The negative flocculation and turbidity tests in these instances were thus most helpful from the differential diagnostic standpoint.

C. Stricture: In no instance in these ten cases was more than one of the four tests incorrect in any one patient.

D. Cirrhosis: The results listed in Table IX indicate that the individual tests were frequently misleading in the twenty-six cases of cirrhosis. In Case

98 all four of the procedures under discussion yielded results that were misleading as judged by the arbitrary standards. This patient did have a cirrhosis as proved by the surgical biopsy specimen. The patency of the bile duct was established by cholangiography at the time of surgery. The patient was believed to represent an example of cholangiolitic cirrhosis.[14] Further evidence of the regurgitation of bile was the elevated blood bile acid: patient, 3.8; control, 0.7 mg. per 100 c.c. as determined by the method of Josephson.[15]

Four cases (Cases 93, 95, 97, and 99) gave results that were misleading with three of the four tests. These results were particularly disturbing in Cases 95 and 99 because both the thymol turbidity and the cephalin-cholesterol tests gave normal values and the phosphatase levels were high. Both patients were middle-aged women with jaundice of long standing and cirrhosis of unknown etiology. The patency of the bile ducts was established in each instance by subsequent exploratory operation. The cirrhotic process was not due to biliary stricture or ascending cholangitis so far as could be determined. In the reremaining two instances (Cases 93 and 97) the thymol turbidity alone behaved in the expected fashion. Necropsy performed in Case 93 revealed a finely nodular, 2,625 gram liver that was classified as a portal cirrhosis of the Laënnec type by the pathologist. The bile ducts were patent; the gall bladder had been previously removed surgically. In Case 97 the patient had had jaundice and pruritus of six years. A biopsy diagnosis of cirrhosis had been made previously and the patency of the bile duct established by operation. Although the cholesterol and phosphatase values were not remarkably elevated, the blood bile salt level was (patient, 4.1; control, 0.7 mg. per 100 c.c.). Clinically this patient also was believed to represent an instance of choangiolitic cirrhosis.

In ten cases in the cirrhotic group, two of the four tests proved to be misleading. In two of these, Cases 79 and 82, this was especially serious in that both the thymol turbidity and the cephalin-cholesterol tests gave negative results. Thus biochemically they closely resembled the previously mentioned six cases of common duct stone that failed to show the expected elevations of cholesterol and phosphatase. The other eight cases were characterized mainly by misleading elevations of the cholesterol or phosphatase (Table VI).

E. Hepatitis: The figures listed in Table IX show considerable deviation from the anticipated results in the thirty-one cases of hepatitis. The results are slightly superior to those obtained in cirrhosis however, possibly because this is a more homogeneous group. The criticism may be made that we have employed certain biochemical tests to establish a diagnosis of hepatitis and then have proceeded to use this diagnosis to demonstrate the value of the tests. This weakness in such a study is appreciated, but in the absence of any specific biologic tests for virus hepatitis, all such diagnoses, in the absence of a biopsy, must be based on clinical features and course.

In two cases in this group, the results of these four tests were quite misleading in that three of the four procedures gave results more in keeping with the arbitrary standards listed for extrahepatic obstruction. Case 108 (Table VII) failed at any time to show a positive result by either the Hanger or the

Maclagan tests and the initial cholesterol level was 230; serial observations showed a rise to 398 mg. per 100 c.c. and an eventual decline to 225. Yet, the clinical course appeared to leave little doubt that the jaundice was due to hepatitis. Complete recovery ensued after a three-week illness; subsequent examination by cholecystography revealed a normally functioning gall bladder. Case 129 (Table VII) was very similar in regard to these tests. The patient was followed by serial tests at intervals of one week. The tests performed during the fifth and sixth weeks revealed total serum bilirubin levels of 2.6 and 1.5 mg. per 100 c.c. respectively. On these two occasions only, the cephalin-cholesterol test was 2 plus and the thymol test 6 units. Subsequent values were again normal and complete clinical recovery ensued.

In six of the cases of hepatitis, two of the four procedures gave misleading results. In three of these (Cases 112, 116, and 124) the cholesterol and the phosphatase were elevated rather than normal or decreased as might be expected in hepatitis; the abnormal turbidity and flocculation tests were therefore of considerable diagnostic value in these two instances. Cases 131 and 134 failed to demonstrate cephalin-cholesterol values greater than 1 plus at 24 hours and the total cholesterol values were higher than might be anticipated in hepatitis. The thymol turbidity value was only 2 units and the alkaline phosphatase level was 11 Bodansky units in Case 104.

F. Miscellaneous Hepatic Disease: This small group of thirteen patients has been included in this study chiefly because they had sufficient elevation of the serum bilirubin to conform with the criteria previously listed and because the tests under consideration were performed simultaneously. In general, the clinical nature of their illnesses was such that tests of liver function were not employed particularly for differential diagnosis. If judged arbitrarily, however, the results of the tests were misleading in most instances. The serum cholesterol showed to best advantage in this group. The low value in Case 147 was particularly striking. At necropsy, this 27-year-old woman was found to have a markedly fatty liver as a complication of severe and intractable ulcerative colitis of three months' duration.

DISCUSSION

The purpose of this study was to evaluate the merits of what has been termed a "minimal yet adequate" program of liver function studies in the differential diagnosis of jaundice.[4] For this analysis, the program has been minimal since only the initial single set of determinations obtained in each patient has been evaluated. The limitations and merits of the individual tests have been so frequently and thoroughly discussed in current literature that no attempt will be made to review that aspect here. The group of tests employed in this study can be performed on a single sample of serum and technically represent procedures that are well within the range of a modern hospital laboratory. The merits of these procedures in differential diagnosis have been subjected to critical testing on the basis of rather arbitrary standards.

The quantitative van den Bergh reaction when used as a means of determining the bilirubin content of the serum has much to recommend it. The very real clinical value of the test in differentiating retention and regurgitation jaundice has been mentioned. Malloy and Evelyn[16] indicated that the quantitative van den Bergh did yield results that would permit the differentiation of the various forms of regurgitation jaundice, a view shared by Meakins.[17] Ducci[13] indicated, on the basis of his extensive experience in 1,770 cases, that the percentage of the prompt direct reacting bilirubin bore some relationship to the type of jaundice. In hepatocellular jaundice he observed the value to be "over 50 per cent," in incomplete obstructive posthepatic jaundice, "generally over 60 per cent," and in complete obstructive posthepatic jaundice, "generally over 70 per cent." In the present small series of cases the relationship of the degree of biliary obstruction to the behavior of the serum bilirubin has not been studied. It is apparent from Fig. 1 that the levels of the one-minute and the total bilirubin and the percentage relationship were too variable to have diagnostic significance.

The serum cholesterol levels and particularly the level of the cholesterol ester in the jaundiced patient have long been employed to good advantage. We have been repeatedly impressed with the merit of serial determinations of the total and esterified cholesterol in evaluating the severity of liver damage. The occurrence of low values for the total serum cholesterol in jaundice usually indicates severe hepatocellular dysfunction. This is particularly true when there is an associated sharp reduction in the concentration of the esterified fraction.[18-21] The results obtained in the twenty-eight cases of jaundice due to cancer indicate that the cholesteral ester fraction did not increase to the same degree as did the free form. In four of the cases (Table III) the total cholesterol was greater than 225 mg. per 100 c.c. and the ester fraction comprised more than 50 per cent. In nineteen of the cases, though the total cholesterol exceeded 225 mg. per 100 c.c., the ester fraction was below 50 per cent. In nine of these nineteen cases the absolute value for the ester was below 100 mg. per 100 cubic centimeters.

In the instances of calculus jaundice (Table IV), twenty of the thirty-nine patients exhibited total cholesterol values greater than 225 mg. per 100 c.c. and esterified fractions greater than 50 per cent. Seven of these had total cholesterol values greater than 225 mg. per 100 c.c. with less than 50 per cent esterified. However, in only two of these cases was the absolute value for the ester below 100 mg. per 100 cubic centimeters.

On the basis of this limited number of cases, it might be suggested that this difference of behavior of the serum cholesterol ester in cancerous and calculus jaundice has some diagnostic significance. It should be noted that Epstein and Greenspan[19] found elevated cholesterol ester levels as well as elevated total cholesterol levels in 75 per cent of their fifty-two cases of jaundice due to cancer, a finding at variance with earlier reports.[21-22] Reduced serum cholesterol ester percentages in jaundice due to cancer have been recorded by others.[23] This

and other aspects of the serum cholesterol behavior in jaundice do not warrant undue emphasis, however. Snell[24] has indicated the difficulties encountered in attemping such correlations.

The variations of the serum alkaline phosphatase activity in jaundice have been utilized for diagnostic purposes since 1933.[25, 26] Gutman and associates[27] reported that the majority (approximately 90 per cent) of cases of extrahepatic biliary obstruction had levels of alkaline phosphatase activity greater than 10 Bodansky units. In many cases of diffuse liver damage, the alkaline phosphatase failed to exceed 10 Bodansky units. The diagnostic merits of combining the phosphatase determination with the cephalin-cholesterol flocculation test[28] or with the thymol turbidity test[29] have been described. The results of the present study are in agreement with the observations[27] that there is better correlation of elevated alkaline phosphatase in jaundice due to cancer than in that due to calculus obstruction (Table IX). The fact that certain cases of diffuse liver damage have phosphatase values greater than 10 Bodansky units is also well recognized.[27] The results shown in Table IX and in Fig. 3 bear this out.

The present study indicates that the cephalin-cholesterol and thymol turbidity tests, when considered together, were seldom misleading in the surgical cases. In only one instance in the seventy-seven cases of extrahepatic obstruction (Case 2) were both tests simultaneously positive on this single examination. The opposite aspect, that is the failure of the tests to exhibit positive reactions in the presence of parenchymal liver disorders, is more common. The two instances in the hepatitis group in which both tests were negative (Cases 108 and 129) have received comment. There were five such instances in the cirrhotic group and seven in the miscellaneous group.

It seems apparent from recent observations that the zinc turbidity test of Kunkel[30] yields fewer false positive reactions in cases of extrahepatic biliary obstruction than does the thymol turbidity. As Popper and associates[31] have observed, the zinc turbidity test will likely prove to be of additional aid in the laboratory diagnosis of jaundice.

In summary, it may be stated that certain characteristic biochemical responses do occur fairly uniformly as the result of hepatic disease and bile duct obstruction. *Most* cases of extrahepatic bile duct obstruction exhibit elevated cholesterol and alkaline phosphatase levels and have normal responses to the thymol turbidity and the cephalin-cholesterol flocculation tests. *Many* cases of jaundice due to diffuse liver disease exhibit biochemical changes of an opposite nature; consequently, such tests have differential diagnostic value. Certain cases of parenchymal liver disease exhibit changes that closely parallel those seen in extrahepatic obstruction, i.e. elevated cholesterol and phosphatase values and normal responses to flocculation and turbidity reactions. This constitutes a limitation of the use of so-called tests of liver function in differential diagnosis that must be accepted. It is a reflection of a basic pathologic phenomenon: the diffuse liver damage existing in hepatitis and cirrhosis may

manifest itself with varying emphasis on hepatocellular or cholangiolar functional impairment. Hepatocellular functional impairment may be regarded as the classical type and the resultant biochemical changes are quite characteristic and readily recognizable. When cholangiolar functional impairment predominates in cases of hepatitis and cirrhosis, the results of the tests under discussion may prove misleading insofar as differential diagnosis is concerned.

CONCLUSIONS

1. The results of five biochemical procedures (bilirubin, cholesterol, phosphatase, cephalin-cholesterol flocculation, and thymol turbidity) applicable to a single sample of serum have been analyzed as to their respective merit in the differential diagnosis of jaundice.

2. The results in seventy-seven patients with jaundice due to extrahepatic biliary obstruction have been contrasted with those obtained in seventy patients with jaundice due to parenchymal liver disease.

3. The results of these tests in jaundice due to extrahepatic obstruction were fairly uniform; nevertheless, deviation from the anticipated results in two or more of the tests occurred in ten instances.

4. The results of these tests in jaundice due to parenchymal liver disease were variable. They were adequate from a diagnostic standpoint in the cases where hepatocellular dysfunction predominated. In a significant number of cases, cholangiolar dysfunction apparently predominated and the results of the tests resembled those seen in extrahepatic biliary obstruction.

REFERENCES

1. Hoffbauer, F. W., Evans, G. T., and Watson, C. J.: Cirrhosis of the Liver: With Particular Reference to Correlation of Composite Liver Function Studies With Liver Biopsy, M. Clin. North America **29**: 363, 1945.
2. Watson, C. J., and Hoffbauer, F. W.: Liver Function in Hepatitis, Ann. Int. Med. **26**: 813, 1947.
3. Schwimmer, D., Klotz, S. D., Drekter, I. J., and McGavack, T. H.: A Fasting-Blood-Sample Procedure in the Differential Diagnosis and Management of Hepatic Disease, Am. J. Digest. Dis. **12**: 1, 1945.
4. Shay, H., and Siplet, H.: Minimal Yet Adequate Program of Liver Function Studies in the Differential Diagnosis of Jaundice, Am. J. Med. **4**: 215, 1948.
5. Popper, H., and Steigmann, F.: Differential Diagnosis Between Medical and Surgical Jaundice by Laboratory Tests, Ann. Int. Med. **29**: 469, 1948.
6. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119**: 481, 1937.
7. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt Reacting and the Choloroform Soluble Types, J. LAB. & CLIN. MED. **30**: 293, 1945.
8. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Investigation **18**: 261, 1939.
9. Maclagan, N. D.: The Thymol Turbidity Test as an Indicator of Liver Dysfunction, Brit. J. Exper. Path. **25**: 234, 1944.
10. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745, 1934.
11. Bodansky, A.: Phosphatase Studies: Determination of Serum Phosphatase Factors Influencing the Accuracy of the Determination, J. Biol. Chem. **101**: 93, 1933.
12. Ducci, H.: The Thymol Test of Maclagan: Standardization and Adaptation to the Evelyn Photoelectric Colorimeter, J. LAB. & CLIN. MED. **32**: 1267, 1947.
13. Ducci, H.: The Contribution of the Laboratory to the Differential Diagnosis of Jaundice, J. A. M. A. **135**: 694, 1947.

14. Watson, C. J., and Hoffbauer, F. W.: The Problem of Prolonged Hepatitis With Particular Reference to the Cholangiolitic Type and to the Development of Cholangiolitic Cirrhosis of the Liver, Ann. Int. Med. **25**: 195, 1946.
15. Josephson, B.: The Determination of Cholic Acids in Blood, Biochem. J. **29**: 1519, 1935.
16. Malloy, H. T., and Evelyn, K. A.: Notes on Operation of the Evelyn Photoelectric Colorimeter, Philadelphia, 1945, Rubicon Company, p. 37.
17. Meakins, J.: Jaundice in Congestive Heart Failure, Mod. Concepts Cardio-vas. Dis. **18**: 37, 1949.
18. Thannhauser, S. J., and Schaber, H.: Ueber die Bezeihungen des Gleichgewichctes Cholesterin und Cholesterinester im Blut und Serum zur Leberfunktion, Klin. Wchnschr. **5**: 252, 1926.
19. Epstein, E. Z., and Greenspan, E. B.: Clinical Significance of the Cholesterol Partition of the Blood Plasma in Hepatic and in Biliary Disease, Arch. Int. Med. **58**: 806, 1936.
20. Greene, C. H., Hotz, R., and Leahy, E.: Clinical Value of Determination of Cholesterol Esters of Blood in Hepatic Disease, Arch. Int. Med. **65**: 1130, 1940.
21. Burger, M., and Habs, H.: Ueber die Veresterung des Serumcholesterins bei Leberkrankheiten, Klin. Wchnschr. **6**: 2221, 1927.
22. Gardner, J. A., and Gainsborough, H.: Blood Cholesterol Studies in Biliary and Hepatic Disease, Quart. J. Med. **23**: 465, 1930.
23. White, F. W., Deutsch, E., and Maddock, S.: The Comparative Value of Serial Hippuric Acid Excretion, Total Cholesterol, Cholesterol Ester, and Phospholipid Tests in Diseases of the Liver: I. The Results of the Tests, Am. J. Digest. Dis. **6**: 603, 1939; II. A Clinical Comparison of the Tests, Am. J. Digest. Dis. **7**: 3, 1940.
24. Snell, A. M.: Unpublished data, in discussion of White, Deutsch, and Maddock.[23]
25. Roberts, W. M.: Blood Phosphatase and the van den Bergh Reaction in the Differentiation of the Several Types of Jaundice, Brit. M. J. **1**: 734, 1933.
26. Bodansky, A., and Jaffe, H. L.: Phosphatase Studies: IV. Serum Phosphatase of Non-osseous Origin, Significance of the Variations of Serum Phosphatase in Jaundice, Proc. Soc. Exper. Biol. & Med. **31**: 107, 1933.
27. Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A.: Effect of Disease of the Liver and Biliary Tract Upon the Phosphatase Activity of the Serum, J. Clin. Investigation **19**: 129, 1940.
28. Gutman, A. B., and Hanger, F. M.: Differential Diagnosis of Jaundice by Combined Serum Phosphatase Determination and Cephalin Flocculation Test, M. Clin. North America **25**: 837, 1941.
29. Maclagan, N. F.: Liver Function Tests in the Diagnosis of Jaundice, Brit. M. J. **2**: 197, 1947.
30. Kunkel, H. G.: Estimation of Alterations of Serum Gamma Globulin by a Turbidimetric Technique, Proc. Soc. Exper. Biol. & Med. **66**: 217, 1947.
31. Popper, H., Steigmann, F., Dyniewicz, H., and Dubin, A.: Use of Thymol Turbidity as Lipid Absorption Test. Experiences With Thymol Turbidity and Zinc Sulfate Turbidity Tests Under Physiologic and Pathologic Conditions, J. LAB. & CLIN. MED., **34**: 105, 1949.

EFFECT OF EPINEPHRINE ON VITAMIN A AND GLUCOSE BLOOD LEVELS IN NORMAL AND CIRRHOTIC SUBJECTS

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THE role of the liver in the regulation of the blood plasma vitamin A level is poorly understood. Although accorded diagnostic significance in hepatic disorders, the plasma concentration does not necessarily reflect the vitamin A concentration in liver tissue.[1-9] On diets restricted in vitamin A, the blood content ordinarily is maintained within the normal range virtually to the point of full tissue depletion. Moreover, although 95 per cent of the body's vitamin A is stored in the liver,[10] the recent work of Sexton and co-workers[11] suggests that this organ may not be, as was hitherto generally believed, the principal site of conversion from the carotene precursor.

Hepatic diseases, however, are often associated with lowered blood vitamin A levels.[12-16] This occurs in cirrhosis, in which the vitamin A content of the liver is greatly reduced.[15, 17] The low plasma levels in cirrhosis might reflect an inability to mobilize the vitamin because of decreased body stores.

Young and Wald[18] noted an increase in the blood vitamin A level of rabbits following epinephrine injection. Observations made in this laboratory indicated that the same phenomenon often occurs in normal human subjects.* This phenomenon was utilized in the present investigation to study the mobilization of vitamin A in patients with cirrhosis. The blood glucose response to epinephrine, which is thought to be a measure of hepatic glycogenolysis[10, 19] and may be otherwise related to vitamin A metabolism,[5, 6, 10, 20, 21, 22] was determined simultaneously.

MATERIAL AND METHODS

Studies were made in twelve patients with cirrhosis of the liver and twenty-two control subjects, in two of whom the procedure was repeated. Two of the twelve cirrhotic subjects had complicating diabetes mellitus and one (with previous jaundice but no clinical diabetes) was discovered at autopsy to have hemochromatosis. The control group—recruited from non-cirrhotic patients, staff members, and medical students—included ten persons with completely normal liver function studies and twelve without clinical evidence of hepatic disease but with one or more, often transitory, abnormal function tests. One of each of these groups was diabetic.

The patients with cirrhosis were on a standard high protein, high calorie diet.[23] Except for appropriate diabetic regimens in two cases there was no regulation of food intake among the control subjects.

The test was performed in the fasting state and consisted of the intramuscular injection of epinephrine (0.6 c.c. of a 1:1,000 solution). Samples of venous blood† were taken before and at one-half, one, and two hour intervals after the injection. Plasma vitamin A (and carotene) levels were determined by the method of Kimble,[24] employing the Carr-Price reaction. Blood sugar concentrations were measured by a modified Benedict procedure.[25]

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*The reciprocal phenomenon, i.e. a decrease in the plasma vitamin A level, likewise has been observed following injection of an epinephrine antagonist, insulin, and, on occasion, following the injection of glucose alone.

†Simultaneous determinations have shown essentially parallel changes in arterial and venous blood plasma vitamin A levels.

RESULTS

Results are shown in Table I.

Following administration of epinephrine, the maximum variation in the direction of initial change is recorded in terms of percentage of the preinjection level of each constituent measured. In the case of vitamin A an increase of 5 per cent or more is arbitrarily regarded as significant, i.e. a positive test. A

TABLE I. BLOOD VITAMIN A, CAROTENE, AND SUGAR LEVELS FOLLOWING THE INTRAMUSCULAR INJECTION OF EPINEPHRINE IN PATIENTS WITH CIRRHOSIS OF THE LIVER (WITH MEAN VALUES FOR THE CIRRHOSIS AND CONTROL GROUPS)

PATIENT	BLOOD SUBSTANCE	BLOOD LEVEL SPECIMEN				PER CENT INITIAL CHANGE
		FASTING	½ HR.	1 HR.	2 HR.	
1	Vitamin A	57	52	50	45	-21
	Carotene	389	385	395	356	- 1
(C)	Sugar	77	80	79	84	+ 9
2	Vitamin A	44	57	59	40	+34
	Carotene	115	115	112	108	- 6
	Sugar	97	103	104	102	+ 7
3	Vitamin A	46	60	48	47	+30
	Carotene	158	162	166	158	+ 5
(D)	Sugar	117	129	142	153	+31
4	Vitamin A	86	82	81	87	- 6
	Carotene	279	303	303	293	+ 9
(C)	Sugar	78	107	99	103	+37
5	Vitamin A	93	93	99	85	- 9
	Carotene	117	108	115	105	- 8
	Sugar	78	93	111	90	+42
6	Vitamin A	114	117	110	120	+ 3
	Carotene	92	97	72	96	+ 8
	Sugar	65	99	103	81	+58
7	Vitamin A	100	109	102	94	+ 7
	Carotene	99	110	107	99	+11
	Sugar	88	99	103	82	+17
8	Vitamin A	107	128	114	114	+20
	Carotene	178	187	128	188	+ 5
(C)	Sugar	80	90	98	97	+23
9	Vitamin A	124	92	91	90	-26
	Carotene	100	96	99	99	- 4
(D)	Sugar	124	218	195	169	+75
10	Vitamin A	32	42	37	48	+50
	Carotene	139	150	100	146	+ 7
	Sugar	65	70	74	75	+15
11	Vitamin A	38	32	31	29	-24
	Carotene	69	69	77	68	+12
	Sugar	80	92	90	90	+15
12	Vitamin A	28	27	20	31	-29
	Carotene	73	74	75	73	+ 3
	Sugar	64	70	76	73	+19
						Range
Cirrhosis group	Vitamin A					+ 2 (-29 to + 50)
	Carotene		Mean:	12 Tests		+ 3 (- 8 to + 12)
	Sugar					+29 (+ 7 to + 58)
Control group	Vitamin A					+ 3 (-17 to + 24)
	Carotene		Mean:	24 Tests		0 (-17 to + 15)
	Sugar					+47 (+ 4 to +156)

(C), Patient receiving carotene.
(D), Patient with diabetes mellitus.

blood sugar elevation of 30 per cent above the fasting figure is regarded as normal—virtually the same value as that generally accepted for the response to epinephrine.[10, 19]

In five of twelve (42 per cent) determinations in the cirrhotic group and in ten of twenty-four (42 per cent) in the control group there was a significant increase in plasma vitamin A after injection of epinephrine. Among the twelve patients with cirrhosis only one showed a significant increase in both plasma vitamin A and blood sugar levels. The vitamin A level alone rose in four others, while a normal blood sugar elevation occurred in four without a positive vitamin A change. Three individuals showed no significant positive shift in either constituent.

Of the control subjects, eight yielded a rise in both blood vitamin A and sugar levels; two, an elevation in the vitamin A alone; eleven, in the sugar alone; and three, in neither substance.

Six patients with cirrhosis and seven of the control group showed decreased vitamin A levels after injection of epinephrine.

Blood plasma carotene levels, measured as a necessary part of the Kimble procedure, showed no consistent changes in either group and served as a check on possible errors due to faulty sampling or variations in blood volume.

There was no apparent correlation between the initial blood concentration and the magnitude of the individual response. However, the most pronounced reductions in vitamin A levels were exhibited among individuals with low fasting levels.

Of the two control subjects in whom the test was performed twice, one exhibited a rise in vitamin A and blood sugar on both occasions and the other showed a vitamin A rise in one and a blood sugar increase in both instances.

DISCUSSION

The plasma vitamin A response to the injection of epinephrine is apparently a variable one. While approximately 42 per cent of individuals exhibit an arbitrarily significant increase in plasma vitamin A concentration, the majority show a trend in that direction and only a few undergo a decrease of comparable magnitude. Since an elevation of plasma vitamin A level occurs with virtually the same frequency among patients with cirrhosis as among normal controls, the response to injected epinephrine in the dose employed cannot be used as a test of hepatic function. Moreover, the frequent failure of control subjects to demonstrate the so-called normal mobilization of blood sugar following injection of epinephrine[10, 19] casts doubt on the validity of this test as well. This finding is at variance with the opinion recently expressed by Kinsell and associates.[26]

Probably most significant is the demonstration that blood plasma vitamin A levels evidently are not a reliable index of body stores since individuals with abnormally low levels may show an increase in the concentration of this substance in response to injections of epinephrine.

SUMMARY

Plasma vitamin A and blood sugar responses to injection of epinephrine were determined in twelve patients with cirrhosis of the liver and in twenty-two control subjects.

A significant increase in plasma vitamin A concentration was observed in 42 per cent of each group. There was no apparent correlation between initial levels and the magnitude of the vitamin A responses. Low fasting concentrations did not preclude a significant increase in plasma vitamin A. There was no correlation between the responses of blood vitamin A and blood sugar to epinephrine administration.

The plasma vitamin A response to epinephrine injection does not appear to be a valid test of hepatic function.

REFERENCES

1. Nylund, C. E., and With, T. K.: Demonstration of Vitamin A Deficiency in Man, Acta med. Scandinav. **106**: 202, 1941.
2. With, T. K.: Studies on the Carotenoid and Vitamin A Content of Serum in Man, Vitamine Und Hormone **1**: 429, 1941.
3. Rosenberg, H. R.: Chemistry and Physiology of the Vitamins, New York, 1942, Interscience Publishers, Inc.
4. Bessey, O. A., and Wolbach, S. B.: The Vitamins: A Symposium of the Council on Pharmacy and Chemistry of the Council of Foods of the American Medical Association, A. M. A., Chicago, 1939.
5. Josephs, H. W.: Factors Influencing Levels of Vitamin A in the Blood of Rats, Bull. Johns Hopkins Hosp. **71**: 253, 1942.
6. With, T. K.: Absorption, Metabolism and Storage of Vitamin A and Carotene, London, 1940, Oxford University Press.
7. Popper, H., Steigmann, F., Meyer, K. A., and Zevin, S. S.: Relation Between Hepatic and Plasma Concentrations of Vitamin A in Human Beings, Arch. Int. Med. **72**: 439, 1943.
8. Stewart, J. D., and Rourke, G. M.: Vitamin A Content of Plasma and Hepatic Tissue Biopsied at Operation; Effects of Preoperative Therapy in Obstructive Jaundice, Surgery **11**: 939, 1942.
9. Glover, J., Goodwin, T. W., and Morton, R. A.: Studies in Vitamin A: Relationship Between Blood Levels and Liver Stores in Rats, Biochem. J. **41**: 97, 1947.
10. Everett, M. R.: Medical Biochemistry, ed. 2, New York, 1946, Paul B. Hoeber, Inc.
11. Sexton, E. L., Mehl, J. W., and Deuel, H. J., Jr.: Studies on Carotenoid Metabolism. VI. The Relative Provitamin A Activity of Carotene When Introduced Orally and Parenterally in the Rat, J. Nutrition **31**: 299, 1946.
12. Horton, P. B., Murrill, W. A., and Curtis, A. E.: Vitamin A and Carotene. I. The Determination of Vitamin A in the Blood and Liver as an Index of Vitamin A Nutrition of the Rat, J. Clin. Investigation **20**: 317, 1941.
13. Haig, C., and Patek, A. J., Jr.: Vitamin A Deficiency in Laennec's Cirrhosis: The Relative Significance of the Plasma Vitamin A and Carotenoid Levels and the Dark Adaptation Time, J. Clin. Investigation **21**: 309, 1942.
14. Haig, C., and Patek, A. J., Jr.: The Relation Between Dark Adaptation and the Level of Vitamin A in the Blood, J. Clin. Investigation **21**: 377, 1942.
15. Moore, T.: Vitamin A and Carotene. XIII. The Vitamin A Reserve of the Adult Human Being in Health and Disease, Biochem. J. **31**: 155, 1937.
16. Popper, H., and Steigmann, F.: The Clinical Significance of the Plasma Vitamin A Level, J. A. M. A. **123**: 1108, 1943.
17. Ralli, E. P., Papper, E., Paley, K., and Bauman, E.: Vitamin A and Carotene Content of Human Livers in Normal and in Diseased Subjects, Arch. Int. Med. **68**: 102, 1941.
18. Young, G., and Wald, G.: The Mobilization of Vitamin A by the Sympathico-Adrenal System, Am. J. Physiol. **131**: 1, 1940.
19. Cantarow, A., and Trumper, M.: Clinical Biochemistry, ed. 3, Philadelphia, 1945, W. B. Saunders Company.

20. (a) DiBella, L.: Action of Carotene and Blood Sugar in Various Animals, Bull. soc. ital. biol. sper. **16**: 352, 1941.
(b) DiBella, L.: Action of Vitamin A on Blood Sugar in Various Animals, Bull. soc. ital. biol. sper. **16**: 353, 1941.
21. DiBella, L.: Transformation of Carotene Into Vitamin A and Action of Carotene and Vitamin A on Glycemia in Various Animals, Arch. sc. biol. Italy **29**: 301, 1943.
22. Wegelin, C.: Antagonism Between Thyroid and Vitamin A and Liver Glycogens, West. J. Surg. **47**: 147, 1939.
23. Patek, A. J., Jr., and Post, J.: Treatment of Cirrhosis of the Liver by a Nutritious Diet and Supplements Rich in Vitamin B, J. Clin. Investigation **20**: 481, 1941.
24. Kimble, M. S.: Photocolorimetric Determination of Vitamin A and Carotene in Human Plasma, J. LAB. & CLIN. MED. **24**: 1055, 1939.
25. Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and Saccharoids (Non-fermentable Copper Reducing Substances), J. Biol. Chem. **92**: 1414, 1931.
26. Kinsell, L. W., Michaels, G. D., Weiss, H. A., and Barton, H. C., Jr.: Studies in Hepatic Glycogen Storage. I. Adrenalin-Induced Hyperglycemia as an Index of Liver Function, Am. J. M. Sc. **217**: 554, 1949.

INTESTINAL PARASITISM IN AMERICAN TROOPS IN GERMANY

Relation to Transmission of Viral Hepatitis

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With the Technical Assistance of Guenther Resemann,
Corporal Jack Cranmer, and Eleonore Vollmer

THE epidemiology of the sporadic, endemic hepatitis now occurring in Germany among American Occupation Troops is not clear, and it is impossible, in the absence of precise laboratory methods, to distinguish clinically homologous serum jaundice (SH)‡ from infectious hepatitis (IH). As the manner of spread of the two infectious agents is different, with consequent difference in methods of prevention and control, it is important to determine which agent is the predominant one in Germany.

It has been shown definitely that the oral-intestinal route may be used to infect human volunteers with the agent of infectious hepatitis (virus IH), by Voegt,[2] by MacCallum and Bradley,[3] and by Havens and co-workers.[4] Whether there are other routes whereby the disease could spread naturally is not known. On the other hand, the virus SH of "homologous serum hepatitis" has not been transmitted by the oral route—with two possible exceptions[6, 7] which American workers[8, 9, 10, 11] have been unable to confirm. It was thought that at least a partial idea of the amount of fecal contamination in the food of soldiers with hepatitis might be gained by surveying the amount of intestinal parasitism present among them and by comparing the findings with those of soldiers in other Army hospitals who did *not* have hepatitis, with presumably healthy soldiers, and with the indigenous German personnel employed in Army messes.

Although as an oblique study of one facet of the epidemiological problem involved, no significant information was acquired, the findings of widespread intestinal parasitism in American troops in Germany seems worth reporting to call attention to the high incidence of parasitism in all groups studied.

MATERIALS AND METHODS

At the Hepatitis Research Center, 220 patients with hepatitis were examined. The patients were in all stages of the disease, from the acute period to convalescence. The diagnosis of hepatitis had been established by the history, the appearance of icterus, and liver function tests. Patients with carbon tetrachloride poisoning with icterus were excluded from the series, as were patients in whom the diagnosis was doubtful or in whom the icterus had been determined to result from cholelithiasis.

From the Hepatitis Research Center, 120th Station Hospital and the 4th Medical Laboratory, European Command, United States Army.

This study was conducted in cooperation with the Commission on Virus and Rickettsial Diseases of the Army Epidemiological Board, Office of The Surgeon General, United States Army, Washington, D. C., with the approval and interest of Maj. Gen. E. A. Noyes, The Chief Surgeon, EUCOM, and Lt. Col. Carl V. Lind, M.C., Commanding Officer of the 4th Medical Laboratory, to whom appreciation is expressed.

A preliminary report of some of this work has appeared in the Medical Bulletin, EUCOM.[12]

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‡The terms SH and IH have been suggested by Neefe[1] to designate homologous serum jaundice and infectious hepatitis respectively.

As a comparable hospital population, the EUCOM Orthopedic Center (to which, as to the Hepatitis Center, patients are sent from all parts of Germany and Austria) was selected, and 116 orthopedic patients without hepatitis were examined. In addition, a survey of 138 patients on all services in the 279th Station Hospital in Berlin (excluding those with hepatitis awaiting shipment to the Hepatitis Center) was made. This hospital was selected because it receives patients chiefly from the Berlin area where the rate of hepatitis is somewhat higher than in the remainder of Germany and is, furthermore, not in a highly endemic area for any particular parasite; while the Orthopedic Center was at Stuttgart where there is a high incidence of ascariasis among the indigenous pouulation. The incidence of intestinal protozoa is quite uniform throughout the American Zone of Occupation. In addition, 190 presumably healthy soldiers from the hospital in Berlin were examined. At the same time, parasitological examinations on the stools of 213 indigenous personnel* employed in the hospitals were made, results of which have been combined with the findings on previous surveys (July, 1947, to April, 1948) of 1,492 indigenous mess personnel from various Army installations all over Germany. Altogether, a total of 644† military‡ and 1,705 indigenous personnel are herewith reported. The composition of the groups examined and the findings are given in Table I.

TABLE I. INCIDENCE OF PARASITISM IN AMERICAN AND INDIGENOUS PERSONNEL IN THE AMERICAN ZONE OF OCCUPATION, GERMANY

GROUPS SURVEYED*	1	2	3	4	TOTALS OF GROUPS 1, 2, 3, 4	5
DATE OF SURVEY	NOV. 1947	APR. 1948	JAN. 1948	APR. 1948		JUL. 1946-APR. 1948
NUMBER OF PERSONS	212	111	134	187	644	1,705
Endamoeba histolytica	35 (16%)	12 (11%)	13 (10%)	14 (7%)	74†(11%)	25%
Endamoeba coli	38 (18%)	32 (29%)	27 (20%)	43 (23%)	140 (22%)	44%
Endolimax nana	81 (38%)	49 (44%)	32 (24%)	39 (21%)	201 (31%)	37%
Iodamoeba buetschlii	21 (10%)	4 (4%)	6 (4%)	8 (4%)	39 (6%)	11%
Dientamoeba fragilis	3 (1%)	9 (8%)	0 (0%)	10 (5%)	22 (3%)	5%
Giardia lamblia	16 (8%)	15 (14%)	14 (10%)	12 (6%)	57 (9%)	8%
Chilomastix mesnili	6 (3%)	2 (2%)	3 (2%)	1 (0.5%)	12 (2%)	3%
Ascaris lumbricoides	2 (1%)	2 (2%)	1 (1%)	2 (1%)	7 (1%)	10%
Trichocephalus trichiura	2 (1%)	2 (2%)	2 (1%)	0 (0%)	6 (1%)	11%
Enterobius vermicularis	4 (2%)	1 (1%)	3 (2%)	0 (0%)	8 (1%)	3%
Hymenolepis nana	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (0.1%)	0.1%
Hookworm	10 (5%)	8 (7%)	6 (4%)	10 (5%)	34 (5%)	0.1%
Strongyloides stercoralis	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (0.1%)	0.1%
Number of infestations	218	138	107	139	602	2,678
Infestations‡ per 1,000 persons	1,028	1,243	798	743	935	1,571
Persons harboring parasites	128 (60%)	71 (64%)	70 (52%)	83 (44%)	352 (55%)	78%

*Group 1, 120th Station Hospital, Army Hepatitis Center, Bayreuth.
Group 2, 387th Station Hospital, Army Orthopedic Center, Stuttgart.
Group 3, Patients, 279th Station Hospital, Berlin.
Group 4, Detachment personnel, 279th Station Hospital, Berlin.
Group 5, Indigenous personnel employed in Army messes.

†Large races only, 20; large and small races together, 23; small races only, 31. Cysts only, 44; cysts and trophozoites, 21; trophozoites only, 9.

‡Parasitized individuals harbored from one to five species.

*Combined in Table I with 1492 indigenous personnel surveyed between July, 1946, and November, 1947.[12] By indigenous personnel here is meant Germans employed in Army installations.

†Twenty persons included in a preliminary report[13] are here omitted because they either gave a foreign home address or gave no home address.

‡Including twenty-five United States civilians and dependents.

Three techniques were employed on each stool: (1) iron-hematoxylin stained smears, (2) zinc sulfate centrifugal concentration, and (3) thick fecal smears, fixed and dehydrated in 95 per cent and 100 per cent alcohol and cleared in xylol. One zinc sulfate concentration, two hematoxylin smears, and two transparent thick smears were examined for each stool. Each person was given a saline purge and requested to submit a warm, soft, unformed stool sample, with a completed questionnaire. One stool sample from each person was examined. The same procedure was followed in all surveys previously conducted on indigenous food-handlers in Army messes.

RESULTS

A tabular presentation of the survey is shown in Table I. The possible effect of disproportionate numbers of persons with previous residence in southeastern states* of the United States and with tropical service in the various groups and the groups in which trophic forms of protozoa might exist are indicated in Table II. While only 50 per cent of the stools obtained at the Hepatitis Center were actually unformed, a large percentage of the formed stools were soft and were promptly delivered to the laboratory. The material obtained at both Bayreuth and Stuttgart was quite comparable with that obtained from indigenous personnel for several previous surveys.

TABLE II. INCIDENCE OF PARASITISM IN RELATION TO THE PERCENTAGE OF PERSONS FROM SOUTHEASTERN STATES AND WITH TROPICAL SERVICE EXAMINED, AND THE NATURE OF THE STOOL SAMPLE EXAMINED

	INCIDENCE (%)	INFESTATIONS 1,000	PER CENT FROM SE STATES	PER CENT WITH TROPICAL SERVICE	PER CENT UNFORMED STOOLS
Stuttgart	64	1,252	45	23	80
Bayreuth	60	1,028	41	16	50
Berlin, patients	52	798	37	18	35
Berlin, detachment personnel	44	743	34	13	24
Mean	55	935	38.8	16.8	45

Table III shows the incidence of parasitism among persons from southeastern states as compared with that of persons from all other states combined. Thirty-two† (13 per cent) of 250 persons who gave a permanent address in a southeastern state harbored hookworm. Fifteen persons (6 per cent of 250) harbored no other parasites.

One hundred and eight (16.8 per cent of 644) persons had served in the tropics. Sixty per cent harbored intestinal parasites. Sixteen (21.6 per cent) of 74 persons who harbored *Endamoeba histolytica* had been in the tropics.

TABLE III. INCIDENCE FOR PERSONS FROM SOUTHEASTERN STATES AS COMPARED WITH PERSONS FROM OTHER STATES

	SOUTHEASTERN STATES (%)	OTHER STATES (%)
Stuttgart	66	62
Bayreuth	69	54
Berlin, patients plus detachment personnel	63	39
Mean incidence	65	47

*Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Virginia, West Virginia, District of Columbia. Arkansas, Oklahoma, and Texas are included because of the presence of hookworm in certain areas.

†One person from California (with tropical service) and one person from the state of Washington (with no tropical service) also harbored hookworm.

The incidence for persons (all groups combined*) who had been in Germany one year or less was 50 per cent; from not less than one year to two years, 55 per cent; and for more than two years, 48 per cent. In a group of 224 persons who were from states other than southeastern and who had not been in the tropics, incidences were, for these respective periods, 51 per cent, 47 per cent, and 55 per cent.

Fifteen (43 per cent) of 35 colored soldiers harbored parasites.

Seven women patients, 6 of whom harbored parasites, are included in the Berlin survey.

Of 633 persons who stated their age, 36.2 per cent were from 17 to 20 years of age; 30.6 per cent, from 21 to 25; 16.8 per cent from 26 to 30; and 16.4 per cent were over 30 years of age. In the hepatitis group alone the percentages in these respective age groups were 40 per cent, 25.7 per cent, 20.6 per cent, and 14.3 per cent, and the percentages harboring intestinal parasites in these age groups were 62 per cent, 69 per cent, 57 per cent, and 53 per cent. For all surveys combined, the percentages harboring parasites in the four age groups were 53 per cent, 62 per cent, 52 per cent, and 43 per cent.

Sixty-five persons (10.1 per cent) of 644 stated they had had, at the time of the stool examination or within the recent past, one or more episodes of diarrhea. Thirty-nine of these 65 were in the group with hepatitis. Thirty-four (52 per cent) of the 65 harbored intestinal parasites. The percentage of persons with a history of diarrhea was, for each group, as follows: hepatitis patients, 18.4 per cent; orthopedic patients, 3.6 per cent; Berlin patients (all services), 5.8 per cent; Berlin detachment, 6.4 per cent. Of those harboring *E. histolytica*, 31.4 per cent gave a history of diarrhea. For other organisms the percentages were: *Giardia lamblia*, 17.6 per cent; *Chilomastix mesnili*, 25 per cent; *Dientamoeba fragilis*, 4.4 per cent; hookworm, 8.8 per cent.

The patients and detachment personnel at Berlin may be considered as representative residents of that particular area. The patients at the Hepatitis and Orthopedic Centers are drawn from all parts of the American Zone of Occupation.

DISCUSSION

As an oblique approach to the problem of the transmission of "infectious hepatitis" in Germany—one aspect of which may be the contamination of food and water with fecal material—surveys for intestinal parasites have been made on 644 healthy and hospitalized American personnel. The incidence of parasitism in the group with hepatitis was 60 per cent and in a group of orthopedic patients in another hospital the incidence was 64 per cent. A third group of hospitalized persons (all services) had an incidence of 52 per cent, and the incidence in a group of hospital detachment personnel was 44 per cent. An average of 55 per cent harbored from one to five species of parasites.

The variations in incidence found are probably attributable more to such variable factors as former residence in the southeastern part of the United

*Persons with hookworm only, in this instance, were regarded as showing negative results, since the acquisition of hookworm in Germany is highly unlikely.

States, tropical service, and, perhaps mainly, the character of the stool sample examined than to any considerable difference in the amount of fecal contamination of food and water to which any one group had been exposed.

There may be a real difference in the incidences for persons from southeastern states and from other parts of the country, partly attributable to hookworm infestations. However, in the group that contained the highest percentage of persons from southeastern states, and which submitted the most suitable material for stool examinations, no difference is to be noted.

The average incidence for 1,705 indigenous personnel was 78 per cent, with parasitized individuals harboring from one to seven species. If the incidence of parasitism among Americans has been affected by environmental conditions in Germany or by contact with indigenous personnel, it is not apparent in the present study. No appreciable increase or decrease in incidence with continued residence in Germany was found.

SUMMARY

1. A survey of intestinal parasitism among American Occupation Forces in Germany and indigenous German personnel is presented and compared with the incidence of intestinal parasitism in patients suffering from acute and chronic hepatitis. No significant difference between patients with hepatitis and other groups was noted.

2. The average incidence of intestinal parasitism among hepatitis patients was 60 per cent, while the average in other hospitalized patients was 55.3 per cent. The average in apparently healthy American soldiers was 44.0 per cent, while in indigenous personnel it was 78 per cent. An analysis of the individual parasites in each group is presented.

3. A discussion of the significance of these findings in relation to other factors is presented.

REFERENCES

1. Neefe, J. R.: Recent Advances in the Knowledge of "Virus Hepatitis," M. Clin. North America **30**: 1407, 1946.
2. Voegt, H.: Zur Aetiologie der Hepatitis epidemica, München. med. Wchnschr. **89**: 76, 1942.
3. MacCallum, F. O., and Bradley, W. H.: Transmission of Infective Hepatitis to Human Volunteers, Lancet **2**: 228, 1944.
4. Havens, W. P., Jr., Ward, R., and Drill, V.: Experimental Production of Hepatitis by Feeding Icterogenic Materials, Proc. Soc. Exper. Biol. & Med. **57**: 206, 1944.
5. Cameron, J. D. S.: Infective Hepatitis, Quart. J. Med. **12**: 139, 1943.
6. Findlay, G. M., and Martin, N. H.: Jaundice Following Yellow Fever Immunization, Lancet **1**: 678, 1943.
7. MacCallum, F. O., McFarlan, A. M., Marshall, J., Badger, T. L., Salaman, M. H., MacLagen, N. F., and Loutit, J. F.: Discussion on Infective Hepatitis, Homologous Serum Jaundice, and Arsenotherapy Jaundice, Proc. Roy. Soc. Med. **37**: 449, 1944.
8. Neefe, J. R., Stokes, J., Jr., and Reinhold, J. G.: Oral Administration to Volunteers of Feces From Patients With Homologous Serum Hepatitis and Infectious (Epidemic) Hepatitis, Am. J. M. Sc. **210**: 29, 1945.
9. Havens, W. P., Jr., Ward, R., Drill, V. A., and Paul, J. R.: Experimental Production of Hepatitis by Feeding Icterogenic Materials, Proc. Soc. Exper. Biol. & Med. **57**: 206, 1944.
10. Havens, W. P., Jr.: The Period of Infectivity of Patients With Homologous Serum Jaundice and Routes of Infection in This Disease, J. Exper. Med. **83**: 441, 1946.

11. Neefe, J. R., Gellis, S. S., and Stokes, J., Jr.: Homologous Serum Hepatitis and Infectious (Epidemic) Hepatitis. Studies in Volunteers Bearing on Immunological and Other Characteristics of the Etiological Agents, Am. J. Med. **1**: 3, 1946.

12. Burlingame, P. L.: Findings on Stool Examinations of Indigenous Personnel in Army Messes, 1946 to 1948. A Preliminary Report, Med. Bull., EUCOM, U. S. Army **5**: 31, 1948.

13. Burlingame, P. L.: The Incidence of Intestinal Parasites in Troops in Relation to the Transmission of Infectious Hepatitis, Med. Bull., EUCOM, U. S. Army **5**: 22, 1948.

PRIMARY HISTOPLASMOSIS WITH RECOVERY OF HISTOPLASMA CAPSULATUM FROM THE BLOOD AND BRONCHIAL SECRETIONS

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DURING the course of studies on the significance of skin sensitivity to fungus antigens of children with acute respiratory infections and pneumonia,[1] we had the opportunity to observe the clinical course and carry out bacteriologic and serologic studies in a case of primary pulmonary histoplasmosis in a 12-year-old colored boy. We believe that this case is of particular significance because it adds further support to increasing reports concerning the benign nature of histoplasmosis and completes the missing picture comprising the initial infection and progress through the phase of healing. The infection was followed from the onset to complete recovery with beginning calcification. As far as we know, this is the first case of proved active primary pulmonary histoplasmosis to be reported in a child who was born and reared in the Chicago area.

CASE REPORT

J. G., a 12-year-old colored boy who has been observed in the outpatient cardiac clinic for the past four years following an attack of rheumatic fever with carditis, was admitted to Sarah Morris Hospital on Sept. 4, 1948, with a tentative diagnosis of recurrent acute rheumatic fever. This patient was born and reared in Chicago and never left Illinois. He developed rheumatic fever and carditis at the age of 9 years. He made a good recovery but was left with mitral insufficiency and some cardiac enlargement. During the past three years he attended a summer camp for cardiac patients and has been in good health. During the years preceding the attack of rheumatic fever he had measles and mumps. The family history was noncontributory except that a sister, 12 years old, died in April, 1944, of rheumatic heart disease. Also a brother, 18 years old, died in March, 1948, with a clinical diagnosis of pulmonary tuberculosis, but no autopsy was done.

Five days before admission, the patient had returned from a summer camp for cardiac patients located near Chicago. On the day of his return he complained of a head cold and headache. He had a slight nonproductive cough and a fever. The day before admission to the hospital he complained of pain in his left elbow, unaccompanied by swelling.

Physical examination revealed a well-developed and well-nourished colored boy of 12 years who did not appear acutely ill. The temperature was 102.6° F., respiratory rate, 28 per minute, and the pulse rate, 82. The positive findings were: moderate nasal congestion, slightly enlarged anterior cervical lymph nodes, slight cardiac enlargement to the left, a loud blowing systolic murmur at the apex which was transmitted to the axilla, and an early diastolic murmur at the apex and base. The lungs were clear, the liver and spleen were not enlarged, and the extremities appeared normal. The laboratory findings are summarized in Table I.

An x-ray of the chest on September 7 revealed clear lung fields and slight cardiac enlargement (Fig. 1). Skin tests done on September 7 with histoplasmin* and tuberculin gave positive reactions with 7 mm. and 13 mm. indurations respectively, while skin tests

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*Fungus antigens were used in dilutions of 1:1,0000. P.P.D. was used in dilution of 0.0001 milligram.

TABLE I. SUMMARY OF LABORATORY DATA

DATE	9/4/48	9/7/48	9/14/48	11/17/48	12/7/48
Red cell count	3.6 mil.	–	4.0 mil.	3.9 mil.	3.9 mil.
Hemoglobin	11.2 Gm.	–	11.2 Gm.	11.9 Gm.	12.6 Gm.
White cell count	5,900	–	5,200	6,500	6,000
Differential count (%)					
Neutrophiles	14	–	50	52	69
Lymphocytes	75	–	38	30	23
Eosinophiles	3	–	1	2	0
Basophiles	1	–	0	1	0
Monocytes	7	–	11	15	8
Urinalysis	Negative	–	–	Negative	–
Sedimentation rate (mm. per hr.)	28	15	–	24	10
Wassermann and Kahn	Negative	–	–	–	Negative
Antistreptolysin titer	--	166	–	100	–
Weltmann reaction	--	5	–	5	–
Blood cultures	Negative	–	–	H. capsulatum isolated	–

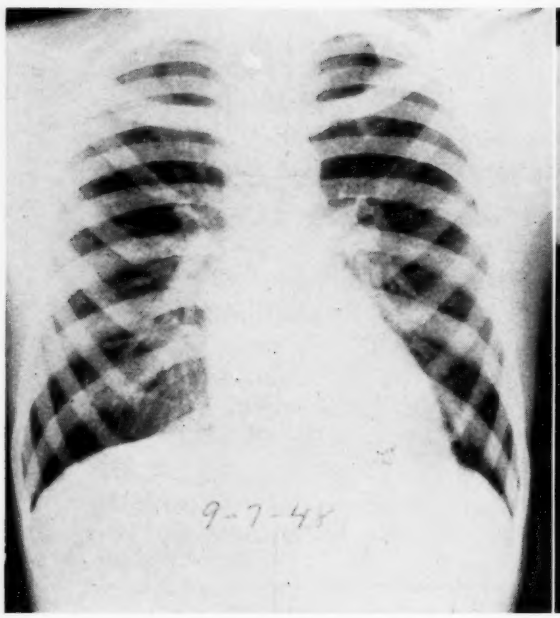
with blastomycin, coccidioidin, and torulin were negative. On symptomatic treatment the patient's temperature became normal after forty-eight hours, and he made an uneventful recovery. There were no significant changes in the cardiac findings. The patient was discharged from the hospital on Sept. 15, 1948, eleven days after admission.

Second Admission.—The patient was readmitted to the hospital on Nov. 17, 1948, with a history of malaise and fever for one day. The temperature was 104° F., pulse rate, 104, and respiratory rate per minute, 22. The blood pressure was 130 mm. of mercury systolic and 62 mm. diastolic. Pertinent findings were injected pharynx, moderate injection of both eardrums, and cardiac findings indicative of mitral stenosis and regurgitation and aortic regurgitation. The pertinent laboratory data are shown in Table I.

The patient was given penicillin and salicylates. His temperature dropped from 104° F. on the day of admission to a maximum of 101° F. on the following day. Subsequently, a low-grade fever up to 100.2° F. persisted for an additional eight days, the total duration of fever being ten days. During this period the patient did not appear ill despite the fact that a large pulmonary infiltration was observed in the left lung on November 18 (Fig. 2). Occasionally he had a slight nonproductive cough. On November 24, physical findings indicative of pulmonary disease were observed for the first time, consisting of slight dullness and increased breath sounds at the base of the left lung. No râles were heard. Although the patient was asymptomatic ten days after admission to the hospital, x-rays of the chest showed exceedingly slow clearing, and at the time of discharge on December 17, there was only about 50 per cent clearing of the pneumonic process (Figs. 3 and 4). An x-ray of the chest taken on Jan. 4, 1949, revealed further clearing and a soft nodular density in the fifth left interspace. Furthermore, a small area of greater density was present in the sixth left interspace which appeared to be beginning calcification (Fig. 5). On March 21, the infiltration had completely disappeared and minute areas of calcification were present in both the fifth and sixth left interspaces (Fig. 6). The last film taken on May 9 showed no significant changes as compared with that of March 21. Skin tests performed on Dec. 1, 1948, with histoplasmin and tuberculin showed 20 mm. and 10 mm. indurations respectively, while tests with blastomycin, coccidioidin, and torulin remained negative.

BACTERIOLOGIC STUDIES

On admission the throat culture was positive for *Staphylococcus aureus,* and no fungi were isolated. Blood cultures were negative. A blood culture obtained on November 17 during the second admission was positive for *Histoplasma capsulatum* in the yeast phase after incubation in beef infusion broth



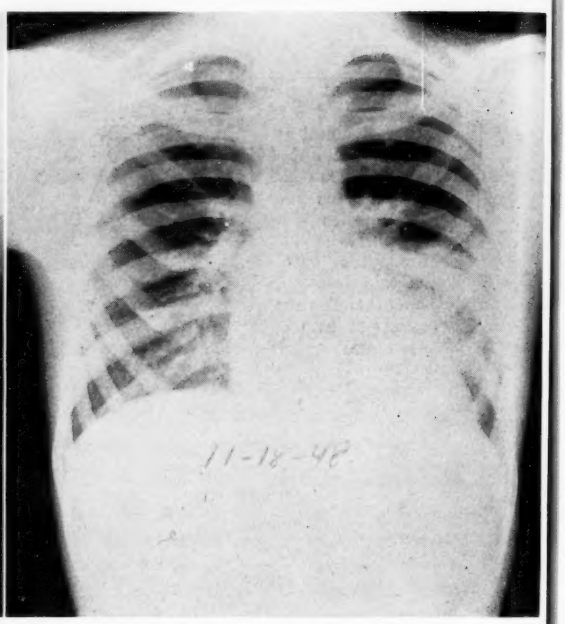
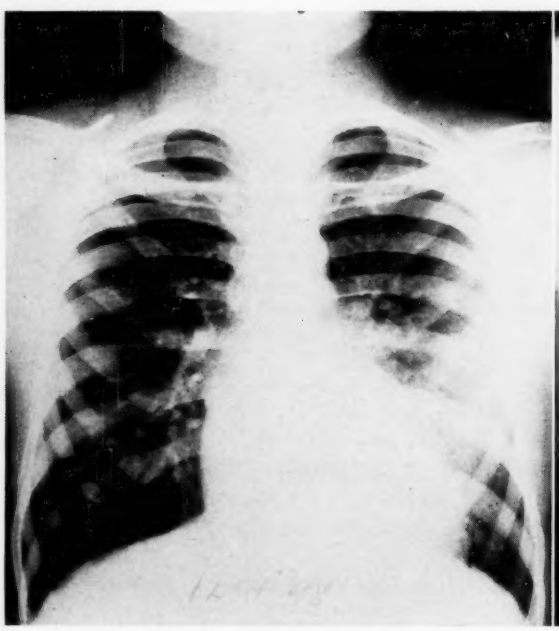


Fig. 1.—Roentgenogram of the chest taken Sept. 7, 1948, at the time of first admission. The hilar lymph nodes appear larger than normal. The lung fields are clear.

Fig. 2.—Roentgenogram of the chest taken Nov. 18, 1948, at the time of second admission. There is a large parenchymal infiltration in the left mid-lung field. There is little change in the appearance of the hilar lymph nodes.



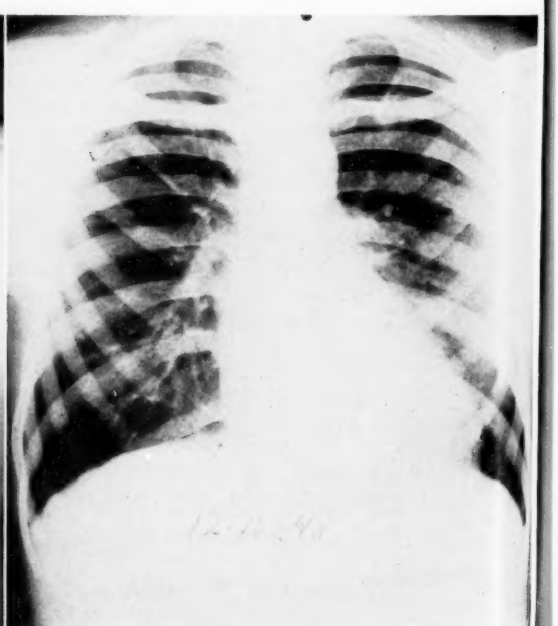


Fig. 3.—Dec. 9, 1948. Since the previous examination of Nov. 18, 1948, there has been about 50 per cent clearing of the infiltrative process in the left lung.

Fig. 4.—Dec. 15, 1948. Since the examination of Dec. 9, 1948, there has been only slight clearing of the infiltration in the left lung.

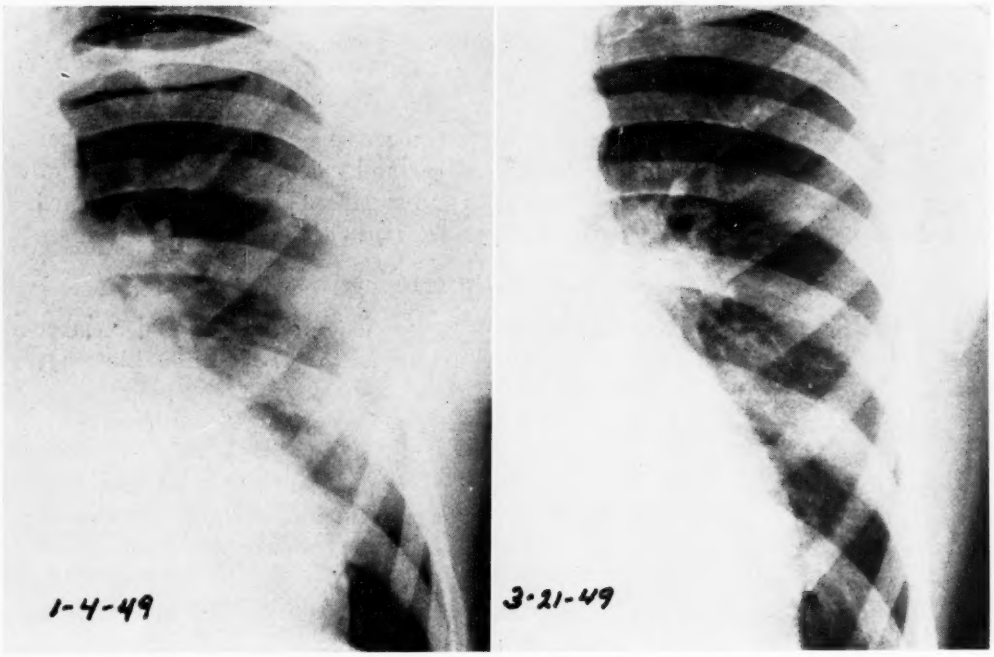


Fig. 5.—Jan. 4, 1949. There is still considerable infiltration in the left mid-lung field. There is an area of focalization in the region of the fifth left interspace. In the sixth left interspace there is a small circumscribed area of greater density that appears to be beginning calcification.

Fig. 6.—March 21, 1949. There is complete clearing of the infiltration in the left lung. Minute areas of calification are present in both the fifth and sixth interspaces. There is an increased amount of calcification in both hilar areas.

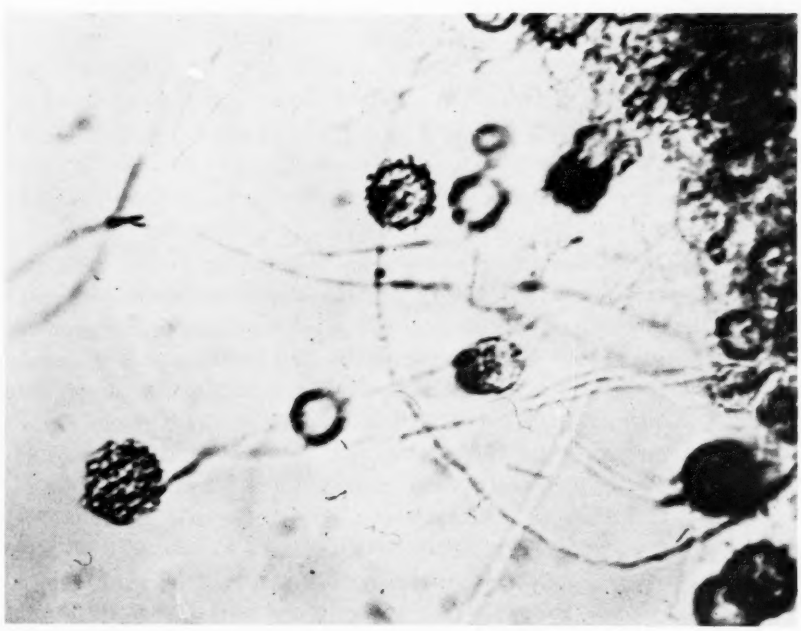


Fig. 7.—Tuberculate chlamydospores of *Histoplasma capsulatum* recovered from the blood on Nov. 17, 1948.

for seven weeks at 37° C. (Fig. 7.) No bacteria were isolated. A throat culture at this time showed anhemolytic streptococci and *Alcaligenes faecalis*. Repeated gastric washings were negative for acid-fast bacilli or pathogenic fungi in direct smear and culture. Bronchoscopic specimens obtained on December 10 were negative for acid-fast bacilli in smear and culture. However, one of six mice inoculated with bronchial material and sacrificed six weeks later showed lesions in the liver and spleen from which *H. capsulatum* was recovered.

SEROLOGIC STUDIES (TABLE II)

Complement fixation tests with undiluted serum carried out on December 1 showed a 4+ reaction with the histoplasmin antigen (lot H-15, 1:100 dilution); on December 20 the reaction was 3+ while on April 2, 1949, the reaction decreased to 2+. The technique described by Tenenberg and Howell[2] and Furcolow and co-workers[3] was employed in the complement fixation tests. Complement fixation tests carried out simultaneously with blastomycin antigen gave negative results. Virus and rickettsia complement fixation tests for psittacosis-pneumonitis group, Q fever, and influenza A and B on some specimens of this sera were repeatedly negative. Serum drawn on December 22 was positive in 1:20 dilution (4+) with lymphocytic choriomeningitis antigen but was negative with serum drawn on April 2. Repeated tests for heterophile and cold agglutinins were negative.

TABLE II. SUMMARY OF VARIOUS COMPLEMENT FIXATION AND OTHER SEROLOGIC TESTS

DATE	11/26/48	12/1/48	12/20/48	12/22/48	4/2/49
Histoplasmin	–	4+*	3+*	–	2+*
Blastomycin	–	0	0	–	0
Psittacosis-pneumonitis	–	–	–	0	0
Lymphocytic choriomeningitis	–	–	–	4+†	0
Influenza A and B	–	–	–	0	0
Q fever	–	–	–	0	0
Cold agglutinins	0	0	–	0	0
Heterophile	0	0	–	0	0

*Undiluted serum.
†Serum diluted 1:20.

DISCUSSION

Although a great deal has been written concerning benign histoplasmosis, pulmonary calcification, and recovery from systemic histoplasmosis, relatively little has appeared describing in detail the previously hypothesized primary phase, which in all probability represents the link between the initial infection and pulmonary calcification. Mention is made of noncalcified pulmonary infiltrates by Dickie and Clark,[4] but these authors failed to describe the initial infection and the progress of the lesions.

Sontag and Allen[5] recently correlated the presence of calcific and precalcific pulmonary lesions with histoplasmin sensitivity in a study of 170 "normal" children. These authors, however, mention no attempt to isolate an etiologic agent. Serial x-rays were made over a period of many months and it was possible to study progressive changes in the lungs from the onset of the initial lesion through the period of calcification. Their studies showed ". . . a

tendency for calcification associated with positive histoplasmin reactions to be: (1) multiple in the parenchyma, often with calcifications present in upper lung fields and apices; (2) early in onset, frequently with the initial lesion appearing before one year of age, the majority before 4 years of age; (3) preceded occasionally by a pneumonic infiltrate early in the infection and (4) progressive with the development of new lesions and calcifications over a period of years.''

In discussing the possibilities of secondary infection with histoplasmosis, Peterson[6] states that in some instances it is almost impossible clinically to differentiate the progressive pulmonary form from pneumonia. Furcolow states[7] that areas of pneumonitis are seen in children in relationship to the development of histoplasmin sensitivity. Although these areas of pneumonitis were often asymptomatic, occasionally they were associated with definite clinical symptoms, especially where there had been marked regional adenopathy and associated pressure reactions. Some of the lesions have the appearance one would expect around a focal granulomatous lesion at the time sensitivity develops. He further states that these lesions are uncommon and that it is often difficult to establish the diagnosis.

In a study of 16,000 school children in Kansas City, Furcolow, Mantz, and Lewis[8] described the roentgenologic characteristics of persistent pulmonary infiltrations in 72 asymptomatic patients who were sensitive to histoplasmin but not tuberculin. They found that only a few were limited to the lymph nodes; a few were of the disseminated type, while approximately two-thirds were nodular, sharply circumscribed foci. The remaining one-fourth were diffuse, patchy infiltrations with poorly defined borders, which sometimes developed into nodular lesions. Similar lesions were seen in a few patients whose symptoms appeared to arise from this infection. In some of the lesions a central core of calcification was noted initially or in subsequent films. The lesions tended to calcify slowly, and many infiltrations persisted without complete calcification during the two years of observation.

These authors also stated that up to the present time they had no cases in which the entire progress of events could be observed, i.e., negative chest x-rays, negative tuberculin and histoplasmin sensitivity through the appearance of the parenchymal infiltrate with concurrent change in cutaneous sensitivity, to the final irreversible calcified focus in the lung or node associated with a positive histoplasmin test (and negative tuberculin test). In all probability a longer period of observation would be necessary in order to observe the final period of calcification. However, our patient began to show calcification approximately 4 months after the appearance of the pulmonary infiltrate. They further state that they have seen patients with initially negative x-rays and histoplasmin reactions who later developed pulmonary infiltrates or hilar adenopathy, or both, together with the development of a positive cutaneous reaction to histoplasmin.

We feel that our case represents the precursor phase of pulmonary calcification and, therefore, completes the hypothetical stage proposed by others. We

were able to demonstrate the progress of the primary phase of histoplasmosis presumably from the onset of the first symptoms and roentgenologic evidence of pulmonary infiltration to final clearing of the lesion and beginning calcification, correlating the clinical progress of the disease with bacteriologic and serologic findings. Radiologic evidence of consolidation was present on Nov. 18, 1948 (Fig. 2), three days after the onset of symptoms and eighty days after the onset of the primary illness (Aug. 31, 1948, when the histoplasmin skin test was positive). It is unfortunate that we failed to do a complement fixation test at this time so that it is difficult to determine exactly when the primary infection took place. Nevertheless, on the basis of the 2+ histoplasmin reaction on November 18 and the subsequent increase in sensitivity associated with the strongly positive complement fixation test, in all probability the primary infection occurred shortly prior to August 31. Thus, one can postulate that the pulmonary infiltration occurred at least eighty-five to ninety days or more from the time of the initial infection.

In our case there may be some question as to the significance of the positive tuberculin test. In all probability there is no correlation between the positive tuberculin and histoplasmin tests. Had we not obtained a positive blood culture and isolated *H. capsulatum* from a mouse inoculated with bronchial secretions, the diagnosis of histoplasmosis and the significance of the positive histoplasmin test would be doubtful.

Fungus skin tests were performed on other members of the family, all of whom are free of pulmonary disease. The mother was histoplasmin and tuberculin positive, and the complement fixation test was negative. The three siblings, 23, 15, and 10 years of age respectively, were positive to tuberculin and negative to all of the fungus antigens. This suggests that in all probability all had a common exposure to tuberculosis.

The complement fixation test apparently is important in distinguishing active and latent or past infections with *H. capsulatum*. Those with recent changes from negative to positive skin sensitivity seem more likely to show positive complement fixation, while those with latent or healed infections are generally negative.[7] The finding of a positive complement fixation test for lymphocytic choriomeningitis (see Table II) was probably due to past infection. No virus was isolated from blood or bronchial secretions obtained during the acute stage and inoculated into mice and guinea pigs. Three other members of the patient's family who were tested at approximately the same time also had positive complement fixation tests for the choriomeningitis virus. The family gave a history of living in a house about six months previously that was overrun with mice. Moreover the complement fixation test became negative when repeated with serum drawn from our patient 101 days after the positive specimen.

SUMMARY

A case of primary histoplasmosis in a 12-year-old colored boy, with clinical findings of atypical pneumonia, is described. The course of the infection was followed from the onset through the stage of pulmonary infiltration to healing

as manifested by beginning pulmonary calcification. *H. capsulatum* was isolated from the blood and bronchial secretions obtained during the acute stage. The patient had a positive complement fixation test for histoplasmosis at the height of the pulmonary infiltration which persisted to the time of beginning calcification four months later. He also had a positive histoplasmin skin test. Benign histoplasmosis is discussed with particular reference to precalcific pulmonary infiltrations.

REFERENCES

1. Whitcomb, Frances C., Milzer, Albert, and Kunstadter, Ralph H.: Incidence of Mycotic Infections in Children With Acute Respiratory Disease. J. Pediat. To be published.
2. Tenenberg, D. J., and Howell, A.: A Complement Fixation Test for Histoplasmosis. I. Technique and Preliminary Results on Animal Sera, Pub. Health Rep. 63: 163, 1948.
3. Furcolow, M. L., Bunnell, I. L., and Tenenberg, D. J.: A Complement Fixation Test for Histoplasmosis. II. Preliminary Results With Human Sera, Pub. Health Rep. 63: 169, 1948.
4. Dickie, H. A., and Clark, E. A.: Histoplasmin and Tuberculin Sensitivity in Relation to Pulmonary Calcification Among University of Wisconsin Students, Ann. Int. Med. 28: 1087, 1948.
5. Sontag, L. W., and Allen, J. E.: Lung Calcifications and Histoplasmin-Tuberculin Skin Sensitivity, J. Pediat. 30: 657, 1947.
6. Peterson, J. C.: Round Table Discussion on Systemic Mycosis, Coccidioidomycosis and Histoplasmosis—American Academy of Pediatrics, Pediatrics 2: 716, 1948.
7. Furcolow, M. L.: Round Table Discussion on Systemic Mycosis, Coccidioidomycosis and Histoplasmosis—American Academy of Pediatrics, Pediatrics 2: 712, 1948.
8. Furcolow, M. L., Mantz, H. L., and Lewis, I.: The Roentgenographic Appearance of Persistent Infiltrates Associated With Sensitivity to Histoplasmin, Pub. Health Rep. 62: 1711, 1947.

LABORATORY METHODS

A NEW MOUNTING FOR THE ELECTROKYMOGRAPH

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IN 1945 Henny and Boone[1] successfully employed an electron multiplier phototube (RCA, 931-A) as a pickup device for the registration of heart motion, using the fluoroscope to delineate the cardiac silhouette. Motion of the heart borders was translated into a varying electric current which was, in turn, recorded as a deflection tracing on an electrocardiograph of either the string or D'Arsonval type. The apparatus was termed an "electrokymograph." Subsequently Henny, Boone, and Chamberlain[2] developed an improved mounting for the phototube which facilitated its use with the ordinary fluoroscope. In 1948 Luisada and co-workers[3] modified this apparatus and called their device a "fluorocardiograph."

In both instruments positioning of the pickup device slit with respect to a given part of the cardiac silhouette is of paramount importance. This was accomplished by both groups through the use of a knurled knob which rotated the phototube pickup by means of a pulley and belt. The knob projected from the operator's side of the screen. In the model used by Henny, Boone, and Chamberlain,[2] the phototube was permanently mounted on a bracket which could be rotated out of the way for routine fluoroscopy. The Sanborn model, employed by us, is constructed according to the modifications developed by Luisada and co-workers and consists of a pantograph arm which clamps to the fluoroscopic screen. Our experience has proved this arrangement to be unwieldy. The phototube tends to rotate after it is positioned and the joints of the pantograph arm give way from the weight of the pickup device. In addition, it was found that the cables of the phototube head frequently dislodged the microphone from its position on the chest when heart sounds were being recorded simultaneously with the electrokymograph.

With these difficulties in mind, we mounted the Sanborn pickup device in the center of an aluminum frame which slides into the existing channels of the 1704 A Picker spot-film device belonging to our x-ray unit. This has the advantage of enabling the entire frame and phototube to be readily slid out of the fluoroscopic field when not in use; when the operator has located the heart border desired, a simple movement of the lead frame holder moves the pickup rapidly into position, and thereafter but minor adjustments of the pickup slit are necessary.

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The Department is supported in part by the Michael Reese Research Foundation.
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*Wedeles Fellow.

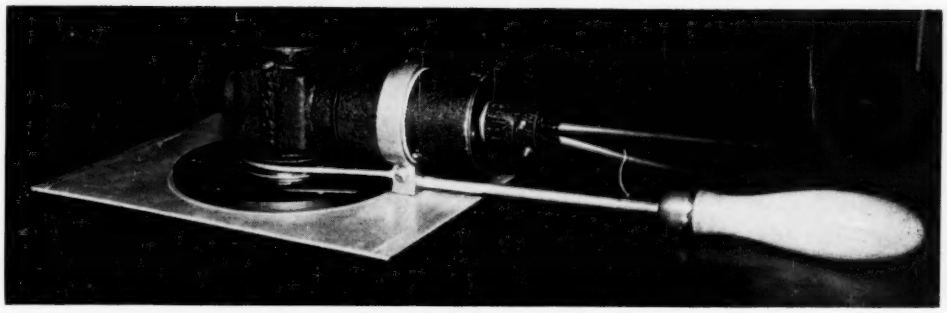

Fig. 1.—A photograph of the electrokymograph pickup device in the new radiolucent aluminum and plastic mounting. Described in text.

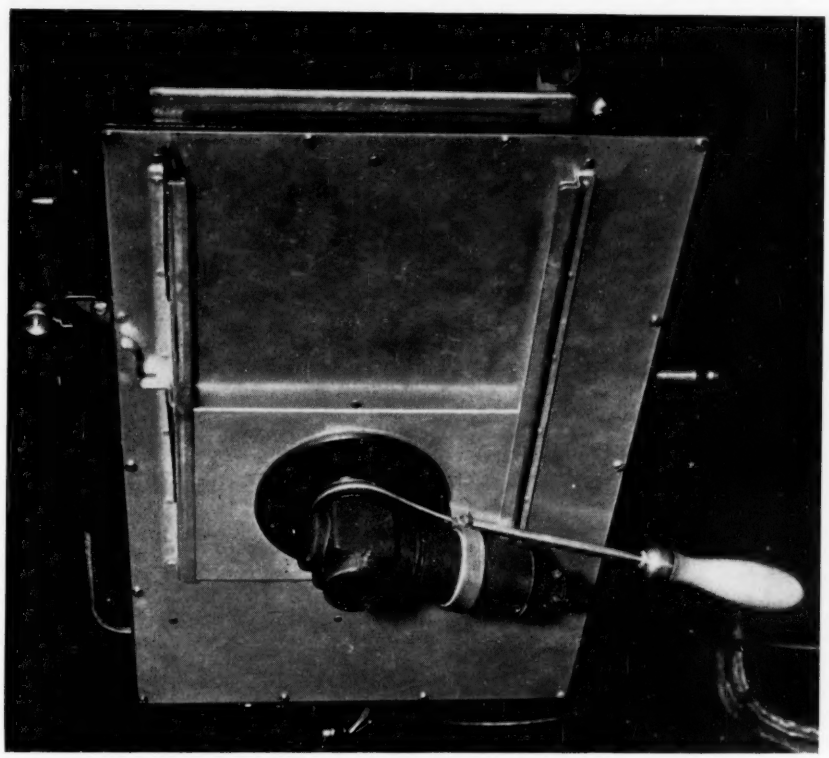

Fig. 2.—The new mounting fitted into the spot-film device.

Positioning of the slit through 180 degrees is accomplished by a long arm (parallel with the slit) similar to that used on a camera mounting. Rotating this arm through a 180 degree arc allows fine orientation of the slit with respect to the cardiac silhouette. Further motion of the phototube is prevented by a screw at the phototube mounting which is operated by turning the handle of the arm. The entire fluoroscopic screen and tube carriage can be locked in any position by the brake on the x-ray unit. Since this is a spot-

radiographic device, it is possible to obtain 5 in. by 7 in. spot films of the phototube pickup head in position on the cardiac silhouette for purposes of permanent record. Figs. 1 and 2 are photographs of this new modification.

SUMMARY

1. A new mounting for the electrokymograph by use of a spot-radiographic device is described.

2. The advantages of this modification are presented.

We are indebted to Mr. N. G. Weidman of Picker X-ray Corporation for valuable suggestions.

REFERENCES

1. Henny, G. C., and Boone, B. R.: Electrokymograph for Recording Heart Motion Utilizing the Roentgenoscope, Am. J. Roentgenol. **54:** 217, 1945.
2. Henny, G. C., Boone, B. R., and Chamberlain, W. E.: Electrokymograph for Recording Heart Motion, Improved Type, Am. J. Roentgenol. **57:** 409, 1947.
3. Luisada, A. A., Fleischner, F. G., and Rappaport, M. B.: Fluorocardiography (Electrokymograph). I. Technical Aspects, Am. Heart J. **35:** 336, 1948.
4. Grossman, N.: The Use of a Spot Film Radiographic Device in Roentgenology, abstract from the Third Inter-American Cardiological Congress, Am. Heart J. **37:** 637, 1949.

METHODS FOR THE DETERMINATIONS OF RADIOACTIVE PHOSPHORUS (P^{32}) IN BODY FLUIDS

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WITH THE TECHNICAL ASSISTANCE OF MARY I. KENDRICK, B.S.

THE use of radioactive isotopes in tracer amounts in man imposes the problem of the determination of very small quantities of isotopes in the body tissues and fluids. This is particularly true when tracers such as P^{32} are used, since the over-all distribution, excretion, and exchange of phosphate are large and rapid. To meet this situation, selection and modification of existing methods for the isolation and estimation of the various phosphate fractions, both radioactive and nonradioactive, are required.

The purpose of the present communication is to present the techniques developed for the investigations of phosphorus metabolism in man using P^{32} as a tracer in doses of 100 to 200 microcuries[1] intravenously.

APPARATUS

The P^{32} beta radiation‖ was counted with end-window (bell type) Geiger-Müller tubes and a Tracerlab Autoscaler. Both commercially manufactured Geiger-Müller tubes and those assembled by the Massachusetts of Technology were employed. The mean window thickness varied from 2 to 5 mg. per square centimeter. The tubes were shielded with 3 to 6 in. of lead. The background varied from 0.23 to 0.45 counts per second for different tubes, but was essentially constant for any one tube during the period of study. For any given set of experiments one tube was used. The critiques of measurement were those reviewed by Kamen.[2]

Calibration of the Autoscaler was made daily by the use of a standard 60 cycle alternating current, and the complete assembly was checked by the use of a uranium standard. A manual sample changer was constructed so that each sample was at a fixed and constant distance from the Geiger-Müller tube window. To ensure maximum recording, this distance was the minimum required to prevent contamination of the tube by the sample.

MATERIALS

Only dried samples were used, and to approximate similar distribution of materials in all samples, the amount of liquid dried was kept constant throughout these investigations. Since 2 ml. was found to be the minimum volume with which accurate counts could be obtained for the least active samples, the more active samples were diluted so that 2 ml. contained an appropriate activity.

Bottle caps,¶ prior to the insertion of the cork inlay, were used as expendable containers for drying samples. Occasionally, such bottle caps were delivered prevarnished. It was found necessary to remove the varnish with a concentrated solution of sodium hydroxide. All bottle caps were washed first with acetone to remove machine oil and grease and subsequently with distilled water and then dried.

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‡The Massachusetts Institute of Technology, and the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital.

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‖P^{32} was supplied by Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tenn.

¶From the Armstrong Company, Lancaster, Pa.

Standards were prepared by drying 2 ml. samples of appropriate dilutions prepared from the solutions of P^{32} which were injected into subjects or added to in vitro reaction mixtures. By means of these, corrections for decay of the isotope could be made.

The method used for drying samples varied depending on the physical characteristics and composition of the substance under investigation. The use of the infrared lamp as a source of heat provided the most convenient and rapid way of drying samples. Unfortunately, it could not be used when the sample contained more than a very small amount of organic matter. When plasma, whole blood, or trichloracetic acid filtrates were dried at a high temperature, the residues were uneven and often elevated, and considerable error in counting resulted. It was found that when samples containing organic matter were dried overnight at 38 to 40° C. more evenly deposited dried samples, closely adhering to the sample containers, were obtained.

Great care must be employed to be certain that the samples are thoroughly dry, since the presence of even small amounts of moisture has been found to lower the counting rate significantly. After most of the moisture had been removed at 38 to 40° C., heating under an infrared lamp or in a hot oven at about 100° C. for one to two hours could be carried out to ensure complete dryness, without causing irregularities in the deposited material.

The thickness, consistency, and density of the samples are important factors in the accurate estimation of radioactivity because of their well-known effect on the absorption of the emitted beta rays. Ideally, all phosphorus-containing fractions should be separated by suitable chemical methods and then converted by chemical procedures to inorganic phosphorus so that direct comparisons of the radioactivity could be made on the same final substance. However, with the use of tracer amounts of P^{32} in man, separation and conversion to inorganic phosphate by organic combustion would introduce dilutions so great as to reduce the radioactivity in some of the fractions of phosphorus to the point where unfeasibly long periods of time would be required for accurate counting. Therefore, no attempt was made to convert all fractions to the inorganic form. As a result, measurements were made on dried samples of different physical characteristics. This was dictated by the necessity of using the maxmium amounts of material in the samples consistent with ease in preparation and counting. Since the samples consisted of rather large amounts of material in nonstandard geometry and unknown uniformity, the corrections for selfabsorption and other physical properties were made empirically. These measurements were also used as a check upon the reproducibility of the data. These corrections will be discussed below in detail for each fraction.

METHODS

Oxalated blood samples, cooled in ice, were centrifuged as soon as possible after the blood was drawn, and the plasma removed as completely as possible. The red blood cells were washed once with ice-cold isotonic sodium chloride solution, centrifuged again, and the supernatant washings removed as completely as possible and discarded. The washed red blood cells were frozen to prevent hydrolysis of phosphorus compounds and to produce hemolysis. Plasma and thawed hemolyzed red blood corpuscles were analyzed chemically for total phosphorus, total acid-soluble phosphorus, and inorganic phosphorus, and the radioactivity of these phosphate fractions was determined. Corresponding values for the total organic, acid-soluble organic, and nonacid-soluble organic fractions were calculated.

Quantitative volumetric measurement of samples of hemolyzed red blood cells was somewhat difficult because of their high viscosity. Probably the most accurate way of measuring them is by weight. This technique is indeed necessary if undiluted red blood cells must be used. However, as a technique for handling many samples of red blood cells, weighing is extremely time consuming. Accordingly, the thawed red blood cells were measured with Ostwald-Folin pipettes calibrated to deliver by blowing out the last drop and transferred to volumetric flasks of desired capacity. The red cells adhering to the sides of the pipette were removed by repeated washings with distilled water; the washings were added quantitatively to the volumetric flask containing the pipetted cells, and the mixture was made up to the desired volume with distilled water. Samples from dilutions of five times the original volume of red cells can be pipetted accurately without difficulty and with good duplication of results.

1. Total Phosphorus in Plasma and Red Blood Cells.—The total phosphorus concentration of plasma and red blood cells was determined in samples of suitable size by the colorimetric molybdate method, after digestion with sulfuric and nitric acids, as described by Fiske and Subbarow.[3]

The total P^{32} content of plasma was determined by counting the beta rays from 2 ml. samples of plasma dried overnight at 38° C.

For the determination of the P^{32} content of erythrocytes, a 1:5 dilution of hemolyzed red blood cells was prepared, and 2 ml. samples of this dilution were dried overnight at 38° C. The beta particles emitted were then counted.

2. Total Acid-Soluble Phosphorus of Plasma and Red Blood Cells.—To precipitate the plasma proteins, 1 vol. of plasma was mixed with 4 vol. of 10 per cent trichloracetic acid. The mixture was shaken well and filtered through Whatman No. 42 filter paper.

In precipitating the protein from red blood cells, a 1:10 dilution was made instead of the 1:5 dilution used for plasma, in order to permit adequate washing of the pipette used to measure the cells. Five milliliter samples of hemolyzed red blood cells were pipetted into 50 ml. volumetric flasks. The pipette was thoroughly washed with water, and the washings were transferred quantitatively to the same volumetric flask. Ten milliliters of 20 per cent trichloracetic acid were added with shaking. The contents of the volumetric flasks were made up to 50 ml. with distilled water, mixed thoroughly by inversion, and filtered through Whatman No. 42 filter paper. This preparation contained the same weight of trichloracetic acid per milliliter of red blood cells as was used per milliliter of plasma, but the final dilution was twice as great in respect to both red cells and trichloracetic acid. Since it was difficult to adjust the volume accurately because of foam and floating particles of precipitated protein, two preparations of red blood cells were routinely made and a sample was taken from each filtrate for all determinations made on the protein-free filtrates.

The total acid-soluble phosphorus in the trichloracetic acid filtrates prepared from plasma and red blood cells was determined by the colorimetric molybdate method of Fiske and Subbarow.[3]

To determine the P^{32} present in the acid-soluble phosphate fractions, 2 ml. samples of the trichloracetic acid filtrates obtained from both plasma and red blood cells were transferred to bottle caps and dried overnight at 38° C. The emitted beta particles were then counted.

3. Inorganic Phosphorus in Plasma and Red Blood Cells.—The inorganic phosphorus of the plasma and red blood cells was determined in the protein-free trichloracetic acid filtrates by the colorimetric molybdate method of Fiske and Subbarow.[3]

For the measurement of P^{32} in the inorganic phosphate fraction of plasma and red blood cells, the inorganic phosphate was separated from the other forms of acid-soluble phosphate by the calcium precipitation method of Fiske[4] with certain modifications and precautions which were found necessary for the final counting techniques.

Twenty milliliter samples of the trichloracetic acid filtrate, prepared as given in the section on acid-soluble phosphorus, were pipetted into 25 ml. volumetric flasks. One or 2 drops of phenolphthalein were added and the filtrate was carefully neutralized with, first, 20 per cent, then normal, and finally, $\frac{1}{10}$ normal sodium hydroxide until a small drop of the $\frac{1}{10}$ normal alkali sufficed to produce a faint pink color. If the end point was passed with one of the stronger alkali solutions, trichloracetic acid was added until the solution was colorless, and the end point established again with $\frac{1}{10}$ normal alkali. *It was found to be of the greatest importance to avoid an excess of alkali because of subsequent failure to obtain complete solution of calcium phosphate.*

The neutralized filtrate was made up to exactly 25 ml. in the volumetric flasks and thoroughly mixed. Two 10 ml. samples were measured into conical, graduated, 15 ml. centrifuge tubes. One tenth milliliter of 0.4 per cent sodium phosphate (approximately 0.1 mg. of phosphorus) was added to the solution in the centrifuge tube to act as a carrier for the P^{32} during subsequent manipulations.

Five milliliters of a 10 per cent solution of calcium chloride, saturated with calcium hydroxide, were then added to the contents of the centrifuge tubes and the mixture was allowed to stand for ten minutes to ensure complete precipitation of the phosphate as calcium phosphate.

The tubes and contents were then centrifuged for two minutes at 3,000 revolutions per minute. *This speed of centrifuging was found to be absolutely necessary for the quantitative separation of the precipitate from the supernatant liquid.*

The supernatant fluid was cerefully decanted from the precipitate and the tubes were drained by standing them inverted on filter paper for a few minutes.

The precipitate was washed once by resuspending in a mixture of 4 ml. of distilled water and 1 ml. of the $CaCl_2$-$Ca(OH)_2$ reagent and recentrifuged at 3,000 r.p.m. for two minutes. The supernatant liquid was decanted and the tubes again were drained by inversion.

Two drops of normal sulfuric acid were added to the calcium precipitate. The gelatinous precipitate of calcium phosphate was replaced by a white amorphous precipitate of calcium sulfate. Repeated tests have shown that all of the phosphate ion at this stage is in solution.

The contents of the 15 ml. centrifuge tube were made up to 5 ml. with distilled water, stoppered, and thoroughly shaken. The stoppers were removed and the tubes centrifuged for about five minutes at approximately 2,000 revolutions per minute.

Two milliliter samples were pipetted from the supernatant fluid into bottle caps and dried. Because of the low salt content and the absence of organic matter in the samples, drying was done rapidly under an infrared lamp. The emitted particles from the dried samples were then counted.

4. Urine.—Urine samples were collected for various intervals of time depending on the periods of most interest in each individual in vivo experiment. The total volumes were measured, and 2 ml. samples of urine were dried and counted with the Geiger-Müller counter and Autoscaler. Following the injection of 100 microcuries of P^{32}, the urine of normal subjects was found to contain enough radioactive material so that 2 ml. samples, diluted or undiluted, gave counts within an accurate range for five or six days following the administration of the isotope. In the first hours the counts were so high that dilution was carried out in order to avoid counting errors while maintaining the 2 ml. volume of sample.

TABLE I. STATISTICAL ANALYSIS OF COUNTING DRIED AQUEOUS SOLUTIONS OF P^{32}

MEAN* (C.P.S.)	STANDARD DEVIATION (C.P.S.)	MEAN* (C.P.S.)	STANDARD DEVIATION (C.P.S.)
1.50	±.07	28.8	±.46
1.61	.11	28.9	.52
2.74	.05	29.1	.43
4.40	.19	29.5	.70
4.45	.19	30.1	.32
4.65	.14	30.5	.64
4.69	.23	32.6	.18
5.10	.18	33.7	.30
8.04	.25	34.9	.96
9.5	.26	39.3	.86
11.8	.14	42.3	.67
12.2	.27	43.3	1.17
12.4	.40	44.1	1.18
12.5	.36	44.1	.95
14.0	.00	45.4	.86
14.9	.59	52.3	.59
15.5	.82	67.3	2.24
16.5	.24	101.7	2.45
18.9	.72	104.7	1.86
18.9	.86	120.6	4.39
19.8	.80	210.8	4.04
21.0	.51	220.0	5.50
21.1	.63		
21.4	.95		
24.2	.64		
25.9	.94		
26.4	.77		
28.0	.51		

C.P.S., Counts per second.
Standard deviation of the mean for the entire series is ± 3 per cent.
*Each figure in the columns "Mean (C.P.S.)" is the mean of six readings, three on each of two duplicate samples.

Correction Factors.—

Folin[5] recommended that 2 per cent should be subtracted from the values obtained for the chemical estimation of total acid-soluble and inorganic phosphate of the plasma in order to correct for the volume occupied by the protein precipitate. This correction was applied to both the chemical determinations and to the counting rates observed for these two plasma fractions.

It was determined experimentally that the protein from red blood cells occupied approximately one tenth of the total volume in the dilution used. Accordingly, 10 per cent was subtracted from the chemical results and also from the counting rates obtained for total acid-soluble and inorganic phosphorus in the red blood cells.

As pointed out earlier, additional corrections were necessary for some of the radioactive preparations because of errors in counting introduced by the physical characteristics and geometry of the dried samples. In addition, all results obtained with a Geiger-Müller counter and Autoscaler are subject to certain intrinsic instrumental inaccuracies.

Dilutions of aqueous solutions of radioactive sodium phosphate were prepared from which duplicate 2 ml. samples were pipetted into the same type of bottle caps used in all subsequent experiments. After the samples were thoroughly dried, each of the duplicates was counted three times. In the first column of Table I are given the means of the six readings taken on each of the fifty samples. The standard deviation from the mean of the entire series, after correcting for background, was approximately ± 3 per cent.

TABLE II. EFFECT OF VARYING AMOUNTS OF ALBUMIN ON P^{32} COUNTING RATE

WT. OF ALBUMIN SAMPLE (MG. PER CM.^{2})	PER CENT OF COUNTS "LOST"*
5.2	0
8.2	0
10.5	0
13.1	3
15.9	4
18.5	5
21.0	7
27.3	9
40.4	15
52.9	18
66.7	23
79.6	26
92.4	30
105.0	34

*All figures are based on the average of duplicate determinations, three observations being taken on each of the duplicates.

TABLE III. EFFECT OF VARYING AMOUNTS OF TRICHLORACETIC ACID ON P^{32} COUNTING RATE

WT. OF TRICHLORACETIC ACID SAMPLE (MG. PER CM.^{2})	PER CENT OF COUNTS "LOST"*
1.3	0
1.7	0
3.4	0
5.2	1
8.5	2
11.3	2
14.0	5
15.9	2
17.8	3
18.2	6
23.7	5
24.4	6
27.0	15
29.8	9
37.5	18
44.0	24
52.7	27
71.5	30

*All figures are based on the average of duplicate determinations, three observations being taken on each of the duplicates.

Additional errors, most likely introduced by the thickness and uneven surfaces of the dried samples, were present. Kamen[2] has stated that the beta rays from P^{32} may show significant absorption due to thickness of the sample film when the weight of the sample exceeds 15 to 20 mg. per square centimeter. Table II shows the corrections necessary when known amounts of P^{32} were added to varying concentrations of human serum albumin. The samples were measured into weighed bottle caps, thoroughly dried, and reweighed. From physical measurements the area of the bottle caps was estimated to be 4.9 cm.^{2}, and the milligrams per square centimeter of albumin were calculated for each sample. In Table III correction factors derived from a similar experiment with trichloracetic acid are given. In both cases, as expected, the counting rate decreased as the thickness of the dried films increased. These results will be discussed later.

RESULTS

Total P^{32} in Plasma.—To determine the correction factor which should be applied to the results obtained for total P^{32} in plasma, a series of twenty samples was prepared containing plasma to which known amounts of P^{32} were added in vitro. The dilution necessarily introduced by the addition of P^{32} solution was kept as small as possible and never exceeded 2 per cent of the total volume. The readings were compared with those of 2 ml. amounts of aqueous solutions containing the same concentrations of P^{32} as the plasma preparations. Each sample was counted at least three times. The results obtained by counting the emissions from the aqueous preparations were regarded as 100 per cent recovery. Statistical analysis of the results showed the average recovery in the plasma samples to be 89 per cent with a standard deviation of ±3.95 (Table IV). On the basis of the recovery experiments, a correction factor of +11 per cent was applied to

TABLE IV. RECOVERY OF P^{32} IN PLASMA
(1.98 ML. OF PLASMA IN 2 ML. SAMPLES; 0.02 ML. AQUEOUS P^{32} SOLUTION)

PER CENT RECOVERY*	PER CENT RECOVERY
87	86
87	93
92	93
92	81
91	82
87	90
86	91
89	91
90	89
92	98

Mean recovery = 89 per cent
Standard deviation = ±3.95

*Each figure is based on the average of at least three observations.

all counts observed for total P^{32} in plasma. Two milliliters of normal human plasma when dried were found to weigh on an average about 34 mg. per square centimeter. Table II shows a loss of 12 per cent of added P^{32} in the presence of albumin of equivalent weight, which agrees closely with the recovery experiments presented in Table IV.

Total P^{32} in Red Blood Cells.—The weight of 2 ml. of a 1:5 dilution of red blood corpuscles was found to be approximately 21 mg. per square centimeter. From Table II it might be predicted that about 94 per cent of the radioactivity

present would be detected. To study this point more thoroughly, twenty-eight in vitro recovery experiments were performed in which 2 ml. samples of red blood cells diluted 1:5 and containing known concentrations of P^{32} were dried and counted. Each sample was counted at least three times. The average recovery of the added P^{32} was 95 per cent (standard deviation of ±3.9) of that observed for dried aqueous solutions containing the same concentrations of P^{32}. (Table V.) This is in excellent agreement with the expected recovery. Consequently, a correction of 5 per cent was applied to the observed counts for total P^{32} in red blood cells.

TABLE V. RECOVERY OF P^{32} ADDED TO RED BLOOD CORPUSCLES DILUTED 1:5 WITH DISTILLED WATER

PER CENT RECOVERY*	PER CENT RECOVERY
99	93
99	99
98	96
93	90
94	94
95	93
92	95
90	98
92	97
90	98
96	99
105	99
88	96
89	94
Mean recovery = 95 per cent Standard deviation = ±3.9	

*Each figure is based on the average of at least three observations.

P^{32} in the Total Acid-Soluble Phosphate Fraction of Plasma.—To determine the necessary correction factor for the measurements of P^{32} in the total acid-soluble phosphate fraction of plasma, thirty-two samples were prepared containing known amounts of P^{32} and 0.16 Gm. of trichloracetic acid in 2 milliliters. This concentration of trichloracetic acid was the same as that of the plasma filtrates. Each sample was counted at least three times. The average recovery was found to be 83 per cent with a standard deviation of ±7.48 as compared with aqueous dilutions containing the same amount of P^{32} (Table VI). Two milliliter samples of the trichloracetic acid filtrate prepared from plasma were found to weigh about 37 mg. per square centimeter. Table III indicated that a recovery of 82 per cent of the added P^{32} would have been expected on the basis of film thickness. Thus, the observed mean recovery agrees well with this value. The fairly large standard deviation is probably due to the very considerable physical differences in the dried trichloracetic acid film. Modifications of this part of the procedure are proceeding.

A series of experiments was performed which indicated that filtration through Whatman No. 42 paper did not contribute to losses in radio activity. Consequently, the observed counts for the total acid-soluble fraction of plasma were corrected on the basis of the in vitro recovery experiments presented in Table VI, which showed an average loss of 17 per cent of added P^{32}, together

TABLE VI. RECOVERY OF P^{32} IN TRICHLORACETIC ACID, 0.16 GM. TRICHLORACETIC ACID IN 2 ML.
(CORRESPONDS TO PLASMA TOTAL ACID-SOLUBLE FRACTION)

PER CENT RECOVERY*	PER CENT RECOVERY
73	84
69	84
85	83
85	78
84	87
64	97
64	78
84	84
83	92
82	84
83	82
82	84
83	89
83	92
83	94
82	94

Mean recovery = 83 per cent
Standard deviation = ±7.48

*Each figure is based on the average of at least three observations.

with the correction of 2 per cent to compensate for the volume of the protein precipitate. Hence, 15 per cent was added to all counts observed for total acid-soluble phosphate in plasma.

P^{32} in the Total Acid-Soluble Phosphate Fraction of Red Blood Cells.—Two milliliter samples obtained from red blood cell protein-free filtrates contained 0.08 Gm. of trichloracetic acid and were found to weigh approximately 17 mg. per square centimeter. Table III shows a recovery of about 97 per cent for samples of this weight. Eighteen recovery experiments containing known amounts of P^{32} and 0.08 Gm. of trichloracetic acid in 2 ml. amounts were counted and found to have an average of 97 per cent recovery of the added P^{32} with a standard deviation of ± 6.1 (Table VII). Again, the fairly large standard deviation is probably accounted for by differences in the physical characteristics of the dried trichloracetic acid film. Since no counts were considered to be more accurate than ± 3 per cent, no corrections were made for absorption due to thickness of film, but 10 per cent was subtracted from all counts observed in the total acid-soluble phosphate fraction of red blood cells to correct for the volume occupied by protein.

TABLE VII. RECOVERY OF P^{32} IN TRICHLORACETIC ACID, 0.08 GM. TRICHLORACETIC ACID IN 2 ML.
(CORRESPONDS TO RED BLOOD CELL TOTAL ACID-SOLUBLE FRACTION)

PER CENT RECOVERY*	PER CENT RECOVERY
110	104
108	100
94	98
93	95
93	90
89	94
100	92
102	92
102	95

Mean recovery = 97 per cent
Standard deviation = ±6.12

*Each figure is based on the average of at least three observations.

P^{32} in the Inorganic Phosphate Fraction of Plasma and Red Blood Cells.—No corrections for loss of P^{32} by selfabsorption were applied to samples of inorganic phosphate. These samples, when dried, weighed about 1 mg. per square centimeter. Sixteen recovery experiments showed an average recovery of 98 per cent of added P^{32} with a standard deviation of ± 4.2 (Table VIII). Two per cent was subtracted from the observed counting rates for plasma to correct for the volume occupied by protein. For the same reason, 10 per cent was subtracted from results obtained for red blood corpuscles.

TABLE VIII. RECOVERY OF P^{32} IN INORGANIC PHOSPHATE FRACTIONS

PER CENT RECOVERY*	
PLASMA	RED BLOOD CELLS
95	103
95	94
90	96
92	101
95	97
94	96
	105
	106
	100
	98
Mean recovery = 98 per cent Standard deviation = ±4.2	

*Each figure is based on the average of at least three observations.

P^{32} in Urine.—Urine samples appeared to offer no difficulties from the standpoint of counting techniques. Their thickness and consistency did not differ enough from that of the control aqueous solutions of P^{32} as sodium phosphate to introduce any significant errors. All of the in vitro tests performed in which known amounts of P^{32} were added to urine indicated 97 to 103 per per cent recovery of the added P^{32}. Some modification of the method might be necessary for urine containing large amounts of protein, but such complications have not been encountered.

It is apparent from the data presented that numerous corrections, principally for selfabsorption, are required in order to make reliable determinations of P^{32} in plasma and red cells by beta particle counting. The correction factors necessary may be determined experimentally, as in the present communication, or selfabsorption correction factors may be approximated from the following equation presented in a simplified theory in an article by Fluharty[6]:

$$\frac{N}{No} = \frac{1}{\mu g}(1 - e^{-\mu g})$$

where No is the counting rate without absorber, N is the counting rate with g grams per square centimeter of absorber, and μ is the mass absorption coefficient.

The relation between μ and the maximum beta ray energy is given roughly (Emax > 0.5 mev) by

$$\mu = 22/Emax^{4/3}$$

where Emax is the maximum energy of the beta rays emitted. Emax is 1.72 mev, and μ is 10.7 for P^{32}.

Fig. 1 presents a plot of the theoretical ratio $\frac{N}{No}$ as a function of milligrams of sample in each square centimeter assuming that the total activity is contained in each sample, but uniformly distributed throughout. Also plotted are the observed values as found experimentally. It may be seen that the

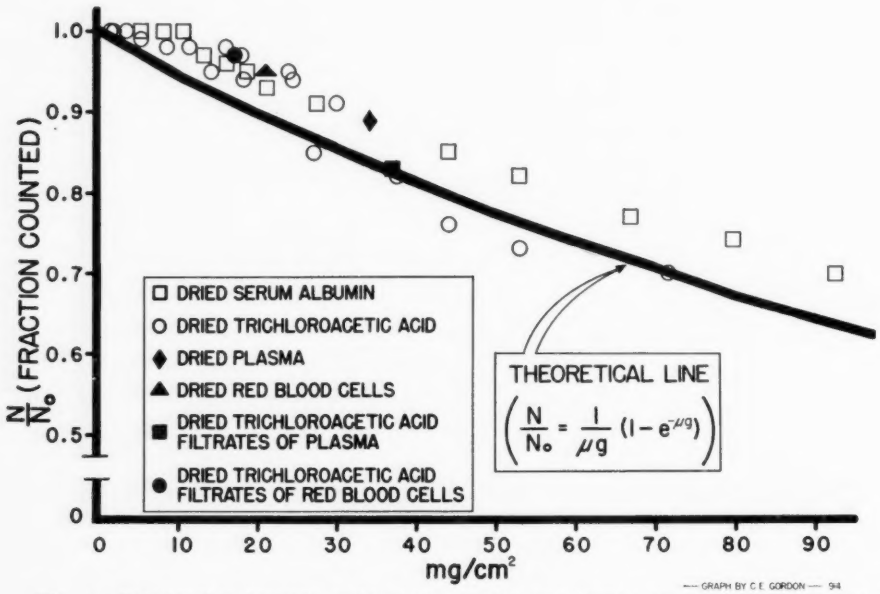

Fig. 1.—Relationship between ratio of "observed" and "true" counts and milligrams per square centimeter sample counted. Solid line represents the theoretical counts based on the relationship between beta ray energy and miligrams per square centimeter, as shown in the equation on the chart.

agreement between theory and experiment is good to within 5 per cent. Except for isolated instances, the fraction of counts observed was greater than the experimental value, but the shape appears proper aside from low quantities of absorbing materials. The discrepancy at low grams per square centimeter is not unexpected, although a satisfactory explanation with experimental proof has not been given. Most likely the variation from theory represents low readings in the standard due to piling up on the edges and subsequent poor geometry with respect to the Geiger tube. The spread in value can be attributed to the differences in the physical character of the different samples.

SUMMARY AND CONCLUSIONS

1. Methods are described for the determination of radioactive phosphorus in plasma, red blood corpuscles, and urine following the intravenous injection of 100 to 200 microcuries of the isotope in normal human subjects. These methods also are applicable to in vitro experiments.

2. The separation of the total acid-soluble and inorganic phosphate fractions of plasma and red blood corpuscles is described and the procedures used for preparing and counting radioactive samples from these fractions are given.

3. Losses of radioactivity by selfabsorption in thick samples are discussed.

4. Certain correction factors are necessary for comparison of counting rates obtained in samples possessing different physical characteristics. The derivation and use of such correction factors are discussed.

REFERENCES

1. Levenson, S. M., Adams, M. A., Fluharty, R., and Taylor, F. H. L.: Studies in Phosphorus Metabolism in Man. III. The Distribution, Excretion and Exchange of Phosphate in Man In Vivo Using Radioactive Phosphorus (P^{32}) as a Tracer. (To be published.)
2. Kamen, M. D.: Radioactive Tracers in Biology, New York, 1947, Academic Press, Inc.
3. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375-400, 1925.
4. Fiske, C. H., and Subbarow, Y.: Phosphocreatinine, J. Biol. Chem. **81**: 629-679, 1929.
5. Folin, O.: Laboratory Manual of Biological Chemistry, New York, 1934, D. Appleton-Century Company, Inc.
6. Fluharty, R. G.: Interaction of Isotopic Radiation With Matter. I. Nucleonics **2**: No. 5, p. 29, 1948.

A METHOD FOR DETERMINING α-AMYLASE ACTIVITY

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UNTIL recently, methods for determining α-amylase activity in serum or urine were based on the estimation of the reducing sugars formed during the enzymic hydrolysis of starch. From the work of Cattle and Hanes[1] it became apparent that the rate of starch hydrolysis could be followed by determining the decrease in starch concentration as measured by the color of the starch-iodine complex. During the last year, while the present work was in progress, two communications appeared[2, 3] which describe methods based on this principle. It is clear from these investigations, and from our own, that α-amylase initially catalyzes the production of starch fragments which yield a different color (or none) with iodine than does starch; that the decrease in starch concentration, as evidenced by the starch-iodine complex color, can be estimated accurately photometrically; and that the decrease in starch concentration initially is a measure of α-amylase activity. Because of the appearance of the latter publications[2, 3] our own experiments concerning the basic principles will not be reported. This paper will be limited to a description of the method developed which seems somewhat simpler than those previously described and appears to be quite suitable for routine work.

METHOD

Reagents.—1. Starch solution. 0.1000 Gm. of Merck soluble starch and 0.3 Gm. NaCl were dissolved in 250 ml. 0.1M $Na_2HPO_4 \cdot KH_2PO_4$ buffer, pH 7.0. This solution cannot be stored for more than one or two days.

2. Iodine solution. 1.27 Gm. iodine and 10 Gm. KI were dissolved up to 1,000 ml. with distilled water. If stored in a glass-stoppered bottle the solution is stable for about one month.

Procedure.—To numbered Klett tubes containing 3.0 ml. ice-cold starch solution are added 0.5 ml. aliquots of whole serum or serum diluted with normal saline. A blank was included. The contents of the tubes are quickly mixed and incubated in a 37° C. water bath for exactly five minutes and then placed in an ice-water bath. Using the same order of addition, 3.0 ml. of the iodine reagent are added immediately and rapidly from a burette. After several minutes, 4.0 ml. distilled water are added and the contents well mixed. In exactly fifteen minutes from the time of addition of iodine reagent the color is read with the Klett-Summerson photoelectric colorimeter using filter 66. It should be noted that the iodine reagent slows amylase action markedly but does not abolish it completely.

RESULTS

To assay the sensitivity of the method and linearity of response, the following experiment was performed. Aliquots of a normal serum were diluted with 0.9 per cent saline as indicated in Table I and the α-amylase activity was determined as described in the previous section. The results indicate that as little as 0.1 ml. of serum can be used and that there is a linear relationship between enzyme activity and starch-iodine color.

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TABLE I. α-AMYLASE ACTIVITY OF DILUTED SERUM

ML. WHOLE SERUM PER 0.5 ML. ALIQUOT OF DILUTED SERUM	OPTICAL DENSITY × 1,000
0	350
0.100	256
0.250	192
0.333	164
0.400	135
0.500	104

If it is desirable, enzyme activity can be expressed in units. One unit is defined as the amount of enzyme in 1.0 ml. which causes the hydrolysis of 0.01 mg. of starch in one minute at 37° C. in a phosphate buffer of pH 7.0. Therefore,

$$\frac{1.2\ (1 - \frac{A}{B})}{0.01 \times 5 \times 0.5} = \text{units}$$

where A is the density reading of the unknown, B is the density reading of the blank, 1.2 is the number of milligrams of starch in 3.0 ml. of substrate, 0.01 is the number of milligrams of starch hydrolyzed per unit activity per minute, 5 minutes is the duration of incubation, and 0.5 is the number of milliliters enzyme solution taken.

TABLE II. REPRODUCIBILITY OF α-AMYLASE ASSAY METHOD

	INEXPERIENCED STUDENT		EXPERIENCED STUDENT	
SAMPLE	DENSITY × 1,000	UNITS	DENSITY × 1,000	UNITS
A	151	27.2	150	27.3
	149	27.5	149	27.5
	149	27.5	153	27.0
B	111	32.8	109	33.0
	103	33.9	104	33.8
	104	33.8	102	34.0
Blank	350		350	

In order to judge reproducibility, the results of a student performing the test for the first time were compared with those obtained by one having experience with the method. The data are summarized in Table II. The time required for the analysis of six samples is approximately thirty minutes.

Sera from several students selected at random were assayed for α-amylase activity. Table III indicates the range of normal values obtained.

TABLE III. α-AMYLASE ACTIVITY OF NORMAL SERA

SUBJECT	AGE	ACTIVITY IN UNITS
Male	28	27.3
Female	23	33.6
Male	26	32.8
Male	24	28.8
Male	27	28.4

SUMMARY

A simple photometric method for determining α-amylase activity has been developed which is applicable as a routine method in the clinical laboratory.

REFERENCES

1. Hanes, C. S., and Cattle, M.: Starch-Iodine Coloration as an Index of Differential Degradation by the Amylases, Proc. Roy. Soc., London, s. B. **125**: 387-414, 1938.
2. Huggins, C., and Russell, P. S.: Colorimetric Determination of Amylase, Ann. Surg. **128**: 668-78, 1948.
3. Smith, B. W., and Roe, J. H.: A Photometric Method for the Determination of α-Amylase in Blood and Urine, With Use of the Starch-Iodine Color, J. Biol. Chem. **179**: 53, 1949.

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A SIMPLE, INEXPENSIVE APPARATUS FOR THE DESICCATION OF BACTERIA AND OTHER SUBSTANCES

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THE advantages of desiccation from the frozen state have been mentioned many times in the literature. The principles involved will not be detailed here, as the reader may find there in many excellent reviews such as those of Greaves[1] and Flosdorf and co-workers.[2]

The apparatus to be described has the following advantages: (1) the device is simply and easily constructed; (2) the cost of operation is low; (3) it is versatile. Material may be dried at various temperatures and to a definite moisture content. The dried material may be sealed off in vacuo or under various gases.

CONSTRUCTION

The apparatus, as seen in the photograph, Fig. 1, is quite simple. It consists of a Pyrex cylinder about 2 in. in diameter, supported horizontally, and of a length to suit the convenience of the operator. It is closed at both ends and has a ground-glass stopper on the top. Five or more side arms of ¼ in. glass tubing extend out on each side at about 10 and 2 o'clock to which are fastened, by means of rubber tubing, the F tubes and ampules. The cylinder will conveniently hold 100 c.c. of sulfuric acid, which is the drying agent.

OPERATION

The sulfuric acid is placed in the apparatus with the aid of a funnel. The stopper is greased with silicone stopcock grease and replaced. The F tubes, ampules, and rubber tubing are also lightly coated with silicone grease and placed on the side arms. The small U-tube mercury manometer is placed on the special arm at one end and the vacuum pump connected to the other. The ampules are frozen in a bath of dry ice (solid carbon dioxide) and alcohol, and the vacuum pump is turned on. When a vacuum of 0.5 mm. or more is obtained, the dry ice and alcohol bath is removed and the material allowed to dry. The liquid in the ampules may be "snap frozen" if dry ice is not available. This is done by cautiously opening the connection between the pump and the machine. Air in solution in the material to be dried will be evolved as bubbles. When all this air is expelled, the connection between the pump and the machine may then be opened completely. The contents of the ampules then will rapidly fall in temperature and within a few minutes will suddenly freeze. The size of the ampule should be large in proportion to the amount of liquid, to prevent bubbles being drawn into the neck of the ampule. After a vacuum has been obtained,

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the pump may be clamped off and disconnected for the duration of the run. The small manometer on one end will indicate any leakage of air. Ordinarily the outfit will hold a vacuum for weeks.

Two precautions are to be taken when using this apparatus. First, if the whole stand is not occasionally gently tilted during the early rapid drying, a

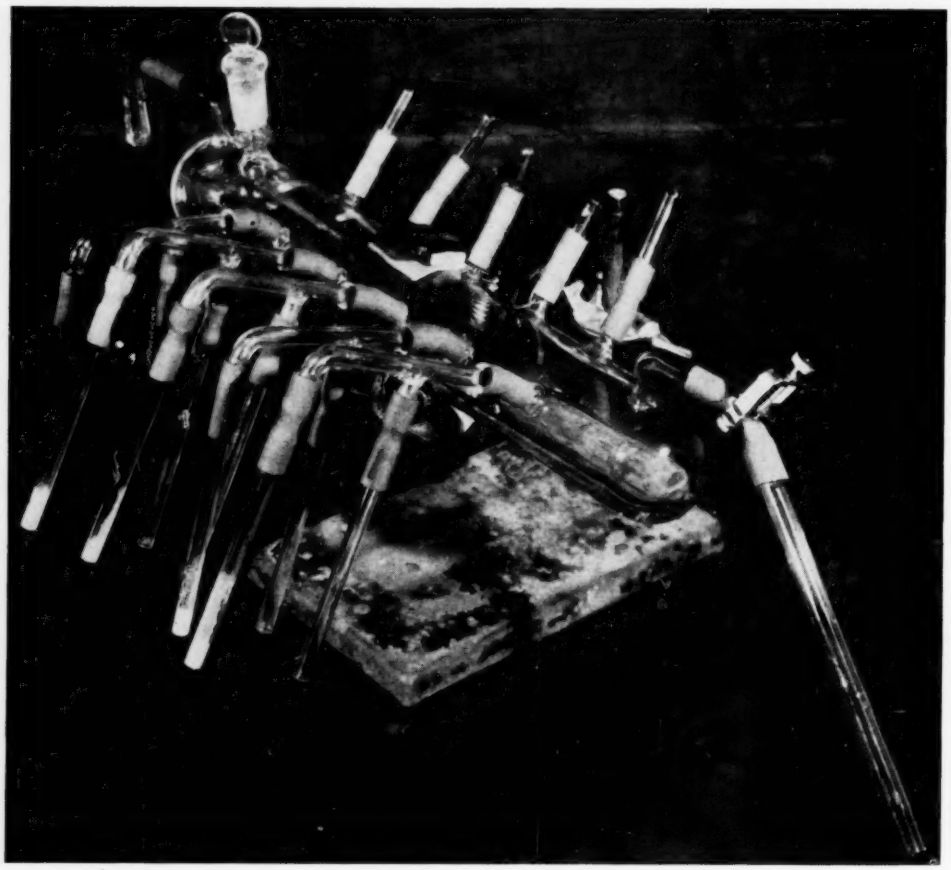

Fig. 1.—Photograph of apparatus.

film of moisture will float on the surface of the acid and reduce the drying speed. Second, the cylinder should be clean. Sulfuric acid is not volatile at ordinary temperatures. However, if the apparatus is not clean, salts such as sodium chloride may react with the sulfuric acid producing volatile acids, such as hydrochloric acid, which may damage the pump.

Material may be dried to a predetermined moisture content by choosing the proper concentration of sulfuric acid. This concentration may be found on the three-dimensional graph, Fig. 2.

This graph should be viewed in perspective. The vertical lines on the graph represent water vapor tension. The numerical values can be read off on the top

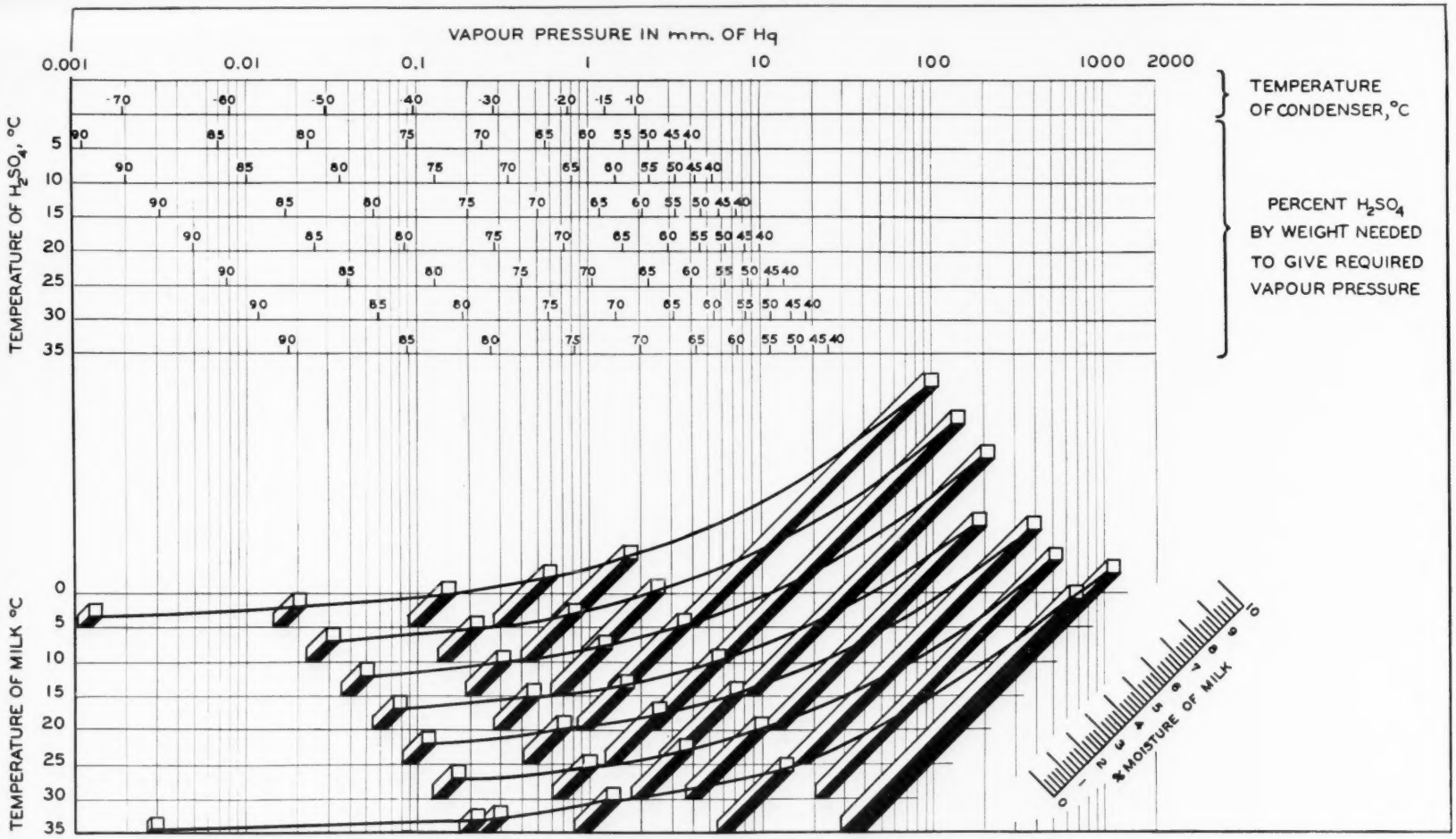

Fig. 2.—Relationship between moisture content of dried milk and vapor pressure. The per cent sulfuric acid necessary for each vapor pressure is also given. Temperature of a cold plate or condenser is also plotted against vapor pressure.

line. The next line indicates the temperatures of a condenser which in a closed system will produce these vapor pressures. The next seven lines indicate the percentage by weight of sulfuric acid necessary at several temperatures to produce these pressures. On the lower left are indicated the final temperatures of the dried milk.

If one wishes to dry milk to a moisture content of, for example, 2.5 per cent at room temperature (25° C.), one chooses a bar which is the same length as the distance from 0 of the moisture scale to the 2.5 mark. This bar should

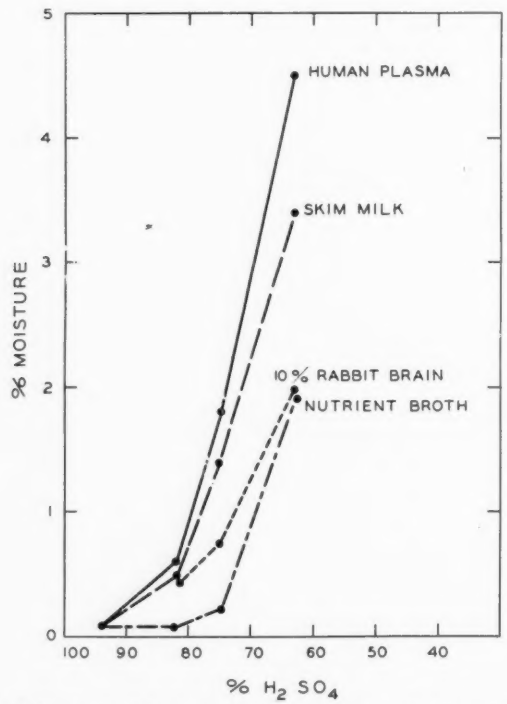

Fig. 3.—Relationship between moisture content of various substances and vapor pressure at equilibrium (three days at 35° C.).

rest on the 25° C. line. Following vertically upward from the base of this bar, we find a vapor tension of 1.2 mm. of Hg. On this same line, we find −15° C., which is the temperature of a condenser giving this vapor pressure. If we wish to use sulfuric acid as a desiccant at room temperature, we find that approximately 69 per cent will be right.

In Fig. 2 the moisture content of milk was determined by placing weighing bottles of dried milk in desiccators over various concentrations of sulfuric acid until the weight remained constant. They were then held over P_2O_5 under a vacuum to constant weight. The loss of weight over P_2O_5 was considered a water loss. Knowing the weight of the sample, the moisture content was calculated. The other data included were obtained from standard tables.[4, 5]

The chart (Fig. 2) was based on skim milk as we have found this a suitable vehicle for drying organisms. The data in Fig. 2 will not apply when predicting the moisture content of other substances dried over sulfuric acid.

As shown in Fig. 3, nutrient broth, skim milk, rabbit brain, and human plasma have been dried for three days over several concentrations of sulfuric acid. Each concentration of sulfuric acid will produce a different water vapor

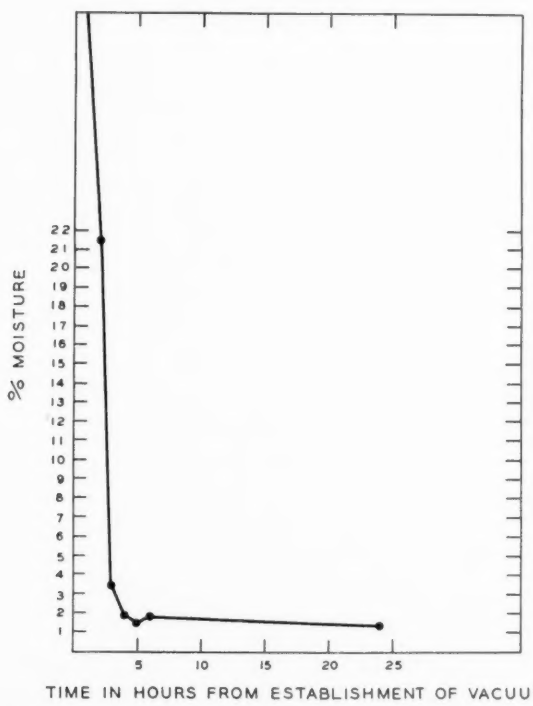

Fig. 4.—Relation between moisture content of milk and time of desiccation on the apparatus described. Moisture contents determined with Fischer reagent.[3] Irregularity at 6 hours probably due to absorbtion of water into ampule which was removed in the evening and tested the next morning.

tension. It is apparent that for any given vapor tension the moisture content of one substance may differ from the moisture content of another.

Desiccation with this apparatus is quite rapid, as illustrated in Fig. 4. It can be seen that equilibrium has been reached in five hours.

Using *Vibrio cholerae* as a test organism, drying runs were made on this apparatus and on another manifold type of apparatus which employed dry ice and Methylcellosolve for moisture removal. On three runs using sulfuric acid, the percentage of organisms remaining viable after drying, as determined by plate counts done before and after drying, was 1.3 per cent, 0.05 per cent, and 0.5 per cent. The percentage of viable organisms on two runs with dry ice was 0.4 per cent and 0.05 per cent.

SUMMARY

An apparatus is described for desiccation of biologicals and other substances which is cheap in initial and operational cost and which will dry to a predetermined moisture content. A graph is presented showing the relations between the moisture content of milk, sulfuric acid concentration, condenser temperature, and vapor tension.

REFERENCES

1. Greaves, R. I. N.: Medical Research Council, Great Britain, No. 258 Special Report, 1946.
2. Flosdorf, E. W., Hull, L. W., and Mudd, S.: Drying by Sublimation, J. Immunol. 50: 21, 1945.
3. Levy, G. B., Murtaugh, J. J., and Rosenblatt, M.: Microdetermination of Water, Indust. & Engin. Chem. Anal. Ed. 17: 193, 1945.
4. Handbook of Physics and Chemistry, ed. 13, Cleveland, Ohio, 1928, Chemical Rubber Publishing Company.
5. International Critical Tables, vol. 3, p. 303, 1928.

ERRATUM

In the article by Innes, Innes, and Moore, "The Hematologic Changes Induced in Guinea Pigs by the Prolonged Administration of Pteroyl Glutamic Acid Antagonists," in the July issue of the JOURNAL, Met-Fol-B was stated to be 7 methyl pteroic acid. That is incorrect. Met-Fol-B is N^{10}-methyl pteroic acid. In the list on p. 885, therefore, and throughout the article, please read N^{10}-methyl pteroic acid instead of 7 methyl pteroic acid.